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*Brit. J. Pharmacol.* (1955), 10, 1.

# THE MODE OF ACTION OF ANTITHYROID DRUGS: FURTHER OBSERVATIONS ON *IN VITRO* INHIBITION OF OXIDATIVE PROTEIN IODINATION

BY

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A preliminary report by Pitney and Fraser (1953) showed that the potency of antithyroid drugs may be assessed by two *in vitro* tests, and that such drugs may differ in their modes of action. In this communication we report further observations with these tests.

The enzyme test—the main test—measures the inhibition by a drug of an enzyme system which iodinates protein. The mechanism of such inhibition may be analogous to that which occurs in the thyroid. The peroxide test measures the ability of the drug to interfere with a non-enzymic but chemically similar iodination of protein. In the tests, two standardized systems are made; one consists of a milk enzyme powder with xanthine, casein and radioiodide, and the other of peroxide, casein and radioiodide. After a standard incubation of the tubes with and without added drugs, the proteins are precipitated and the residual radioiodide in the supernatant fluid is counted to ascertain the degree of protein iodination. These tests are based on the original observation of Keston (1944) that unpasteurized milk with added xanthine will bind radioiodide to its protein, transforming a large proportion to thyroxine, and that this binding is inhibited by thiourea. Keston (1944) postulated that the xanthine oxidase of the milk produces peroxide which releases free iodine and so iodinates the protein. The milk peroxidases are doubtless also important factors in the enzyme reaction of this test. In confirmation of this hypothesis, Pitney and Fraser found that approximately equivalent iodination could be produced in a mixture of iodide and protein by adding either the milk enzymes and xanthine, or peroxide.

The organic binding of iodide by the thyroid gland is generally believed to involve an oxidative mechanism, which may be activated by a peroxidase (Raben and Astwood, 1949; Dempsey, 1944; deRobertis and Grasso, 1946). Without more precise knowledge of the mechanism in the thyroid, it remains uncertain whether this *in vitro* enzyme test is sufficiently analogous; it is, however, encouraging that the test arranges the antithyroid drugs investigated in approximately the order of their biological potency. The test should therefore be useful for a preliminary screening of new antithyroid drugs.

The test also gives some indication of the mode of action of antithyroid drugs. Three main hypotheses have been suggested to account for the inhibition of the organic binding of iodide by antithyroid drugs: (1) that they prevent the liberation of any free iodine, either by their ability to combine with iodine, by their reducing power, or by both (Pitt-Rivers, 1950); (2) that they inhibit (*a*) the thyroid cell peroxidase (Westerfeld and Lowe, 1942; Dempsey, 1944), or (*b*) other oxidative enzyme systems such as cytochrome-oxidase (Schachner, Franklin, and Chaikoff, 1943). The last two hypotheses might be bracketed together as the enzyme-inhibiting theory, since the enzymes concerned in the thyroid are not yet known. Results with the test suggest that the most potent antithyroid drugs may act mainly by inhibiting an enzyme, while many of the weaker drugs may act mainly as substrate competitors or chemical removers of iodine.

## METHOD

### *The Enzyme Test*

The test procedure and the method of preparing the enzyme powder have been fully described by Pitney and Fraser (1953). The dry enzyme powder keeps satisfactorily in the refrigerator. No decrease in potency was noticed during the six months' period of the tests reported here. For these tests 50 mg. of enzyme powder was used in each tube, and, in the absence of drugs, this gave 80% (75% to 85%) protein-binding of iodide. The drugs were compared in terms of molar concentrations. Usually they were tested up to a strength giving 100% inhibition, but sometimes difficulties of solubility prevented the testing of the

full range of concentrations desired—e.g., with 2-thiouracil and *n*-butyl-4-hydroxy-3:5-di-iodobenzoate.

To each of a series of centrifuge tubes are added: 3 ml. phosphate buffer *p*H 7.4, 1 ml. xanthine solution (0.45 mg./ml. just brought into solution in hot alkali), 5 ml. casein suspension (3 g./100 ml. $H_2O$), 1 ml. enzyme solution (50 mg./ml.), 1 ml. iodide solution (iodide $10^{-7}$ M and $^{131}I$ 1 $\mu$c./ml.), and 1 ml. of drug solution. After incubation for half an hour at 37° C., 3 ml. of 25% trichloracetic acid is added; then, after centrifugation, the supernatant fluid is removed and its radioactivity counted. Two pairs of control tubes are run with each test; control A contains neither drug nor enzyme and the supernatant-fluid count gives the figure for 100% inhibition of binding; control B contains enzyme but no drug, and the supernatant-fluid count gives the figure for no inhibition or maximal binding. With the aid of these control values the counts obtained in the other tubes are then each converted to an index of antithyroid activity as follows:

$$\%\ \text{Inhibition of binding by drug in tube X} = \frac{(\text{Count X} - \text{count B}) \times 100}{\text{count A} - \text{count B}}$$

*The Peroxide Test*

The peroxide test is performed similarly except that 1 ml. of 0.1 M-peroxide is added instead of the enzyme and the xanthine. This test need only be used when it is desired to study the mode of action of the antithyroid drug. It is then only necessary to include a pair of tubes containing the lowest concentration of drug found to give 100% inhibition with the enzyme test.

## Results

Fig. 1 shows the results of tests made on a series of known antithyroid and allied drugs; the main findings are summarized in Table I. In the figure, the drugs are compared over a range of concentrations by the percentage inhibition of protein-binding which they induced. These concentrations are expressed as molar equivalents relative to the iodide present in the testing system (molar $\times$ $10^{-8.08}$). The figure shows the results both with the enzyme test and with the peroxide or enzyme-free test. Figures 2a, 2b, and 2c show the curves obtained with each test from three sample drugs. Table I shows various indices derived from these curves for each of the drugs: (1) their potencies with each test (the reciprocal of the molar equivalents for 30% inhibition, comparisons being made at this level, since 50% inhibition was not reached on the peroxide test with some of the drugs); (2) an index of the non-enzymic action in the enzyme test (the percentage inhibition found in the peroxide test with the lowest drug concentration causing 100% inhibition in the enzyme test); and (3) an index of the speed of transition in the enzyme test from minimal to maximal effect with

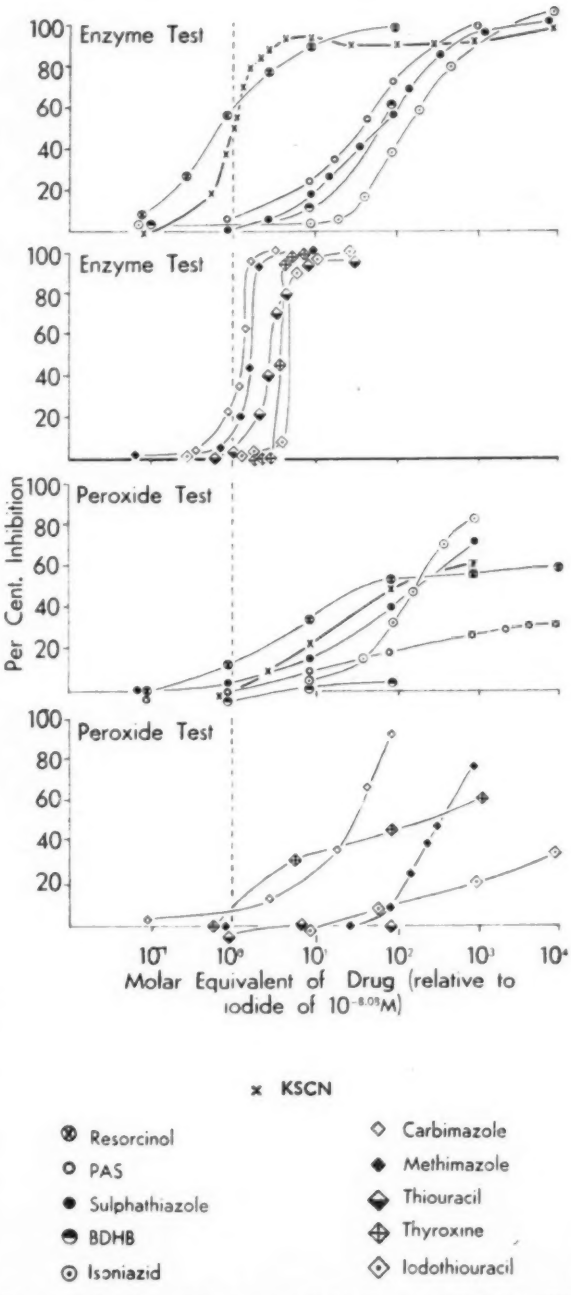



FIG. 1.—Inhibition of *in vitro* protein-binding of radioiodide ($^{131}I$) by various antithyroid drugs. Each curve refers to one drug. The drug concentration is shown in molar equivalents, relative to the iodide present during the test ($10^{-8.08}$M). NOTE: (a) *The enzyme test:* (i) the drug concentration for any chosen level of inhibition arrays the drugs in order of potency, and (ii) the slope of the inhibition curve from minimal to maximal effect varies and indicates different types of action—some, e.g. carbimazole, with steep slopes suggesting enzymic inhibition, and others, e.g. sulphathiazole, with gentle slopes suggesting substrate competition. (b) *The peroxide test:* the degree of inhibition is less and the order of the drugs by potency is different, tending to confirm the indications of enzyme-inhibition by some of the drugs on the former test.

TABLE I

CHARACTERISTICS OF THE INHIBITION BY VARIOUS DRUGS OF THE *IN VITRO* OXIDATIVE PROTEIN IODINATION

| Drug (Mol. Wt. in Parentheses) | Potency (At 30% Inhibition) | | | Non-Enzymic Action in First 100% Enzyme Effect (See Footnote) | Speed of Transition from Minimal to Maximal Inhibition (With Rising Drug Concn.) | | Summary | |
|---|---|---|---|---|---|---|---|---|
| | Enzyme Test | Peroxide Test | Ratio Enzyme: Peroxide | | Enzyme Test (10–90%) | Peroxide Test (10–30%) | Potency | Type of Action |
| Carbimazole (186) | 0·84 | 0·07 | 12·0 | 10% | 0·35 | 0·11 | Strong | E |
| Methimazole (114) | 0·63 | 0·005 | 126·0 | 0 | 0·56 | 0·56 | | E |
| Resorcinol (110) | 2·82 | 0·145 | 19·4 | 55% | 0·011 | 0·10 | | C |
| Thiocyanate (K salt) (97) | 1·12 | 0·056 | 20·0 | 60% | 0·10 | 0·22 | | I |
| Thiouracil (128·2) | 0·35 | — | —‡ | 0 | 0·25 | — | Medium | E |
| Iodothiouracil (276) | 0·18 | 0·0003 | 600·0 | 2% | 0·55 | 0·035 | | E |
| L-Thyroxine (800) | 0·28 | 0·159 | 1·8 | 25% | 0·66 | 0·35 | | I |
| *p*-Aminosalicylate (211·2) | 0·067 | 0·0004 | 168·0 | 28% | 0·005 | 0·003 | Weak | I |
| BDHB* (an antithyroxine) (446) | 0·035 | — | —‡ | Over 5% | Over 0·09‡ | — | | I |
| Sulphathiazole (Na salt) (367·4) | 0·050 | 0·022 | 2·3 | 80% | 0·009 | 0·15 | | C |
| Isoniazid (173) | 0·014 | 0·0126 | 1·1 | 85% | 0·047 | 0·40 | | C |
| Perchlorate (K salt) (138·5) | 0 | 0 | | — | | | Nil | |
| Quinine HCl (400) | 0 | 0 | | — | | | | |

* BDHB = *n*-Butyl-4-hydroxy-3:5-di-iodobenzoate (Sheahan, Wilkinson, and Maclagan, 1951).

‡ Too insoluble for adequate testing (2-thiouracil and BDHB): to derive the figures shown in columns 5 and 6 for BDHB the 50% inhibition values have been used.

E = Enzymic; I = intermediate; C = chemical.

Potency for 30% inhibition = reciprocal of drug concn. for this effect (in molar equivalents relative to the iodide present).

Non-enzymic action in first 100% enzyme effect = % inhibition found on peroxide test with the drug concn. just causing 100% inhibition on enzyme test.

Speed of transition from minimal to maximal inhibition = concn. for lower inhibition (10%) divided by that for higher inhibition (30% or 90%).

rising drug concentration (the ratio of the drug concentration for 10% inhibition to that for a greater inhibition—90% for the enzyme test and 30% for the peroxide test, since with the latter 90% was not always reached).

The drugs have been arranged in Table I in four main groups according to their potency on the enzyme test—high, medium, low and nil. The other characteristics shown in the table have helped to divide the drugs into three main types —enzymic inhibitors (E), chemical inhibitors (C) and drugs with intermediate action (I). In each potency range, the type E drugs have been entered first, since enzymic inhibition may be the optimal type of antithyroid action for clinical use.

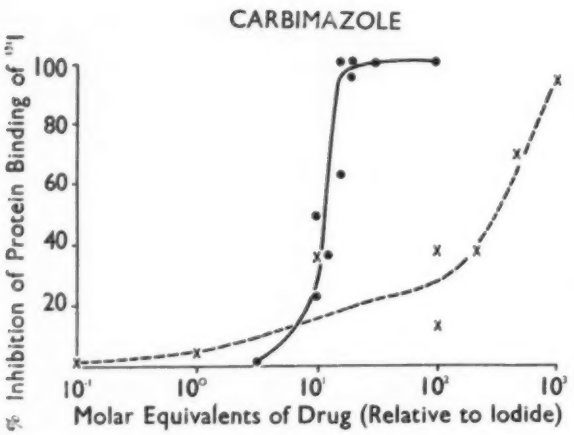
*Potency*

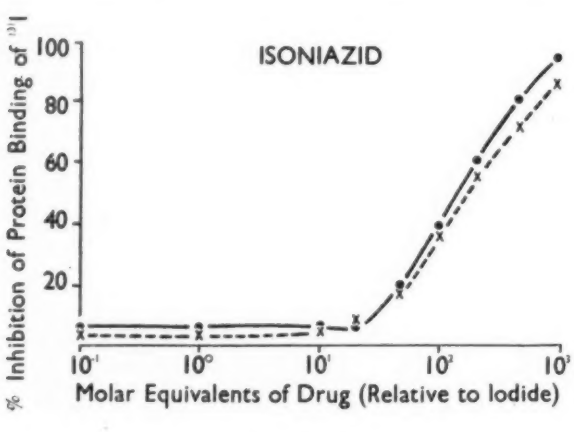
It will be noted that in either test there is little inhibitory action with drug concentrations of less than molar equivalence with iodide ion. Most of the strong inhibitors achieve 100% inhibition in the enzyme test at concentrations between 3 and 10 times molar equivalence. These figures in the enzyme test may suggest the range of concentrations of the drugs which are likely to be required in the thyroid for an antithyroid effect. However, it must be remembered in using these figures that many potent antithyroid drugs are concentrated in the thyroid, while other drugs may not enter the thyroid cell.

It will be noted that all drugs appear more potent by the enzyme test—though the enzyme/peroxide potency ratios for 30% inhibition are far from constant (from 1.1 to 600). Some of this variation might be expected, since the high peroxide concentrations involved in the peroxide test might degrade a number of the drugs (McQuillan, Morton, Stanley, and Trikojus, 1954). The peroxide concentration was chosen to give approximately equivalent protein-binding in the absence of drugs. This is one reason why the peroxide test is not advocated as a reliable index of potency. However, some of the differences between the enzyme/peroxide ratios of different drugs may be due to the different modes of action, and this is suggested by the other analyses shown in Table I and discussed below.

*Type of Action*

Irrespective of potency, there are striking differences in the inhibition achieved in the peroxide test at the concentration which causes 100% enzymic inhibition (" non-enzymic contribution "). This " non-enzymic contribution " is roughly inversely correlated with the " speed of transition " from minimal to maximal inhibition in the enzyme test. The " speed of transition " in the enzyme test is apparently a good inverse index of the " non-enzymic contribution " to the inhibition in the enzyme test, thus confirming the validity of



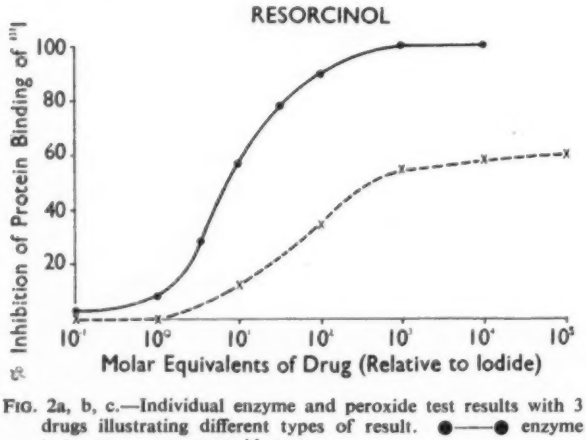




FIG. 2a, b, c.—Individual enzyme and peroxide test results with 3 drugs illustrating different types of result. ●——● enzyme test; ×- - - -×, peroxide test.

this subdivision into types. With thiouracil, *p*-aminosalicylate and resorcinol, the "non-enzymic contribution" is less than would be expected from the "speed of transition." Possibly these drugs are partly destroyed by the high peroxide concentration in the peroxide test. The "speed of transition" is therefore probably the more reliable index of the type of action. By this criterion the drugs have been classed in Table I into three types: *type E*, apparently mainly enzyme-inhibitors with a small "non-enzymic contribution" and rapid "speed of transition" on the enzyme test; *type C*, showing the opposite features and so apparently active mainly by iodine-removal or substrate-competition; and *type I*, showing intermediate characteristics, but resembling more closely type C.

Type E: carbimazole (2-carbethoxythio-1-methyl-iminazole), methimazole (2 - mercapto - 1 - methyl-iminazole), 2 - thiouracil and iodothiouracil (4 - hydroxy-5-iodo-2-mercaptopyrimidine).

Type I: Thiocyanate, L-thyroxine and *p*-amino-salicylate.

Type C: resorcinol, sulphathiazole and isoniazid.

It will be noted that, in general, type E drugs have strong, and type C drugs weak, antithyroid action, both by this enzyme test and by biological tests, with type I drugs more allied to type C. The two main exceptions are thiocyanate and resorcinol, both strong inhibitors on the enzyme test though otherwise resembling type C drugs.

## DISCUSSION

The enzyme test assesses the inhibition of peroxidase-activated iodination of protein. Such peroxidase activity has already been shown to be inhibited by some antithyroid drugs (Westerfeld and Lowe, 1942; Dempsey, 1944). Apart from the identity of the enzyme concerned, conditions in the enzyme test may be comparable to those in the thyroid—the hydrogen ion concentration, the presence of a large excess of protein, and a low iodide concentration. Confirmation of its validity as a test of antithyroid activity can only be final when the enzyme that is active in the organic binding of iodide by the thyroid cells has been isolated. A peroxidase is as likely to be the enzyme involved as any other enzyme suggested (Raben and Astwood, 1949). Direct biological comparison would not be valid, since this also involves the ability of the drugs to enter the thyroid gland and is complicated by differences in sensitivity between various species. It is, however, suggestive that the enzyme test arranges the tested drugs approximately in order of known biological activity, and that no activity has been found with two drugs not known to inhibit the organic binding of iodide by the thyroid (quinine and perchlorate). It is now clear that, in the thyroid, inorganic iodide ion is first concentrated, and then subsequently incorporated into thyroxine (Astwood, 1944–5).

The enzyme test can only be expected to assess activities affecting the second stage. By *in vivo* tests, both perchlorate (Stanbury and Wyngaarden, 1952) and thiocyanate (Vanderlaan and Vanderlaan, 1947) have been found to inhibit the first stage, i.e. concentration of iodide by the thyroid gland, without apparently affecting the second stage. However, this is not discordant with the *in vitro* finding that thiocyanate is an inhibitor, since this drug is known to remain mostly, if not entirely, extracellular in the body. This does, however, emphasize the importance of supplementing this *in vitro* screening test with subsequent biological testing, before drawing final conclusions; for whatever the *in vitro* potency, antithyroid drugs are useless unless they penetrate the thyroid cell.

A theoretically important finding is that antithyroid drugs vary in their mode of inhibiting peroxidase-activated iodination of protein, one group being largely enzyme inhibitors, one group largely substrate competitors or iodine removers, and a third intermediate group showing both types of action. It is probable that drugs similarly vary in their action on the thyroid. Most potent antithyroid drugs rapidly pass from minimal to maximal effect in the enzyme test and have a relatively slight inhibitory action in the non-enzymic peroxide test. Both these features suggest that enzymic inhibition is the major factor in their action in the enzyme test and so presumably in the thyroid. Practically all the antithyroid drugs also inhibit at higher concentrations in the non-enzymic peroxide test. However, the concentrations involved suggest that this may not be their action *in vivo*. The results of the peroxide test must be interpreted with caution, since a much higher concentration of peroxide was used than is likely to be found in the body; some of the drugs tested may not be stable in such a concentration. The results of this test have been presented mainly for the support they give to the interpretation of the enzyme tests. They tend to confirm that the steep curves of the enzyme test indicate enzyme inhibition.

The drugs differ not only in potency but also in their relative inhibitory effect on the enzymic and the non-enzymic tests. Only one group, consisting mostly of the weaker drugs, shows an approximately equivalent effect in both tests and is thus likely to have a mainly non-enzymic action. It appears that antithyroid action may be partly affected by the drug's chemical activity as an iodine-remover or substrate competitor. However, the potent antithyroid drugs also have a more important enzyme-inhibiting action (probably peroxidase-inhibiting). This is contrary to the view expressed by Pitt-Rivers (1950) that the antithyroid action of drugs depends on either an ability to bind iodine or reducing power or both. However, Miller, Roblin, and Astwood (1945) did not find that iodine-removal activity correlated adequately with biological antithyroid potency; nor did Arnott and Doniach (1952) find that the comparative biological antithyroid activity of a series of compounds chemically allied to resorcinol supported this hypothesis.

It is likely that, potency apart, the type E drugs —the enzyme inhibitors—would be the better drugs for clinical use, because their action should involve concentration in the thyroid by attachment to the enzyme. Thus, with an effective antithyroid dose, there should be a lower concentration elsewhere in the body and so a lesser general toxic risk. This test should therefore help in screening new potential antithyroid drugs by picking out from the most potent those which are also enzyme-inhibitors. The two most potent of the type E drugs which we have tested were carbimazole and methimazole—the two drugs reported to give the least toxic effects in a clinically adequate antithyroid dosage (Fraser, Garrod, Hanno, and Jadresic, 1954). Conversely, resorcinol is potent but mainly a type C drug and known to be clinically toxic; and iodothiouracil, found clinically to be merely a weak antithyroid drug, needs about double the molar concentration of 2-thiouracil for equivalent *in vitro* action.

The test should also help to detect undesirable antithyroid action in drugs intended for long-term use for other purposes. This is unlikely to matter with drugs to be used only for short periods, since unless an antithyroid drug is used for longer than three months it will not exhaust the normal thyroid's store of preformed hormone. It will be noted that we found that *p*-aminosalicylate gives 50% inhibition of the enzyme test at about ten times the molar concentration required for thiouracil, or at about thirty times molar equivalence with iodide. Total inhibition does not occur until about thirty times this concentration. If for a rough preliminary assessment we consider an average dose of the drug (5 g.) distributed in 50 litres of body water, its molar ratio to iodide would be about 474:1 (assuming body fluid to contain approximately $1 \times 10^{-6}$ mols. iodide/l.). Thus, ignoring excretion and the unknown possibilities of concentration in the thyroid, an antithyroid effect is possible with this dose given three times daily and might be important clinically after

three months' administration. This has been observed (Balint, Fraser, and Hanno, 1954). Similar rough calculations for other drugs may help to indicate the possibilities of such undesirable antithyroid effects and the need to look out for them during clinical trials.

## Summary

1. Further experience with two *in vitro* tests confirms their value for determining the potency and the mode of action of antithyroid drugs.

2. The antithyroid action of some drugs may be attributed to their chemical ability to remove or compete with iodine, but that of the most potent ones probably involves enzymic inhibition of a peroxidase.

3. For screening possible new antithyroid drugs the enzyme test is advocated, using various drug concentrations so as to give from 0–100% inhibition. Positive results should be confirmed *in vivo*.

4. To be suitable for clinical trial, antithyroid drugs should show enzyme inhibition in this test, and be more potent than currently available drugs.

5. If any drug which is likely to be used for prolonged periods is found to give over 50% inhibition in the enzyme test at concentrations likely to be achieved in the body, a watch should be kept for antithyroid effects during clinical trials.

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*Brit. J. Pharmacol.* (1955), **10**, 7.

# *IN VITRO* TESTS OF CHEMICAL COMPOUNDS ON *ASCARIS LUMBRICOIDES* AND *FASCIOLA HEPATICA*

BY

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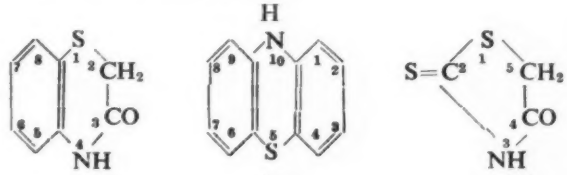
(RECEIVED JUNE 2, 1954)

Baldwin (1948) tested over 200 chemical compounds *in vitro* against *Ascaris lumbricoides* ("roundworm") and found that anthelmintic potency was influenced by the presence of certain groups and by the arrangement of groups within the molecule. Mackie and Raeburn (1952a) found that 2:3-dihydro-3-ketobenzo-1:4-thiazine (I) and a number of its derivatives produced a paralysant effect on *Fasciola hepatica* (liver fluke) *in vitro*, and were able to arrange substituent groups in order of potency. Azo-dyestuffs from 6-amino-2:3-dihydro-3-ketobenzo-1:4-thiazine were either inactive or only depressant when tested *in vitro* against liver fluke and the anterior preparations of roundworm (Mackie and Cutler, 1952).

The present paper describes an extension of this work, to include the *in vitro* testing against roundworm and liver fluke, not only of additional derivatives of (I), but also of derivatives of phenothiazine (II), rhodanine (III), and a number of miscellaneous compounds.



(I) (II) (III)

Derivatives of phenothiazine were tested because it has been so successful as an anthelmintic in veterinary practice, despite certain disadvantages. Compound (III), which contains the -S-$CH_2$-CO-NH- group (cf. I), might be expected to show some activity, and the testing of its derivatives seemed desirable. The miscellaneous compounds tested included those which were promising against the free-living stages of sclerostomes (Parnell and Mackie, 1952).

From a study of these various compounds, some observations on *in vitro* anthelmintic effect and chemical constitution have been made.

## METHODS

*Preparative.*—Many of the compounds were prepared by known methods, but the 2:3-dihydro-3-ketobenzo-1:4-thiazine derivatives and most of the phenothiazine and rhodanine derivatives were either new compounds or prepared by improved methods (Mackie and Raeburn, 1952b; Mackie and Cutler, 1953, 1954; Mackie and Misra, 1954).

*Biological Testing.*—The compounds were tested *in vitro*, employing Baldwin's kymographic technique for roundworms (1943) and Chance and Mansour's modification of this method for liver flukes (1949). Anterior preparations of roundworms were used, since they contain the so-called "nerve-ring" (cf. Baldwin, 1943). Certain details of procedure have already been recorded (Mackie and Raeburn, 1952a).

## RESULTS

*2:3-Dihydro-3-ketobenzo-1:4-thiazine Derivatives.*—Mackie and Raeburn (1952b) stated that this compound, and the derivatives described, had practically no effect on intermediate preparations of the roundworm. Nineteen of the 37 compounds of this type, described in the paper cited, and by Mackie and Cutler (1952, 1953), however, showed depressant effects on anterior preparations. Table I summarizes the effects of the new compounds

TABLE I

*IN VITRO* EFFECT OF 2:3-DIHYDRO-3-KETOBENZO-1:4-THIAZINE DERIVATIVES ON *ASCARIS LUMBRICOIDES* AND ON *FASCIOLA HEPATICA*

(P = paralysant)

| Substituent | Effect On | |
|---|---|---|
| | *Ascaris* | *Fasciola* |
| 6-Chloroacetamido- | − | P (1:3,000) |
| 6-Benzoylamido- | − | + |
| 6-Toluene-*p*-sulphonamido- | − | + |
| 6-*p*-Acetamidobenzenesulphonamido- | − | + |
| 6:7-Diethoxy- | − | P (1:1,000) |
| 6-Bromo- | − | P (1:20,000) |
| | | +(1:100,000) |
| 6-Methyl- | ++ | P (1:5,000) |
| 6-*Tert.*-butyl | − | + |
| Benzo-1:4-thiazine | P (1:2,000) | P (1:1,000) |

active against roundworm or liver fluke, or both (for previous results see Mackie and Raeburn, 1952a; Mackie and Cutler, 1952). In this and other tables, all concentrations are 1:1,000, except for those compounds with paralysant (P) and lethal (L) effects, where the figures in parentheses are minimum effective concentrations. Other effects are indicated as follows: strongly depressant + +; depressant +; little or no effect −.

The derivatives already investigated by Mackie and Raeburn (1952a) had the following effects against *Ascaris*:

*Strongly Depressant*: 6-acetamido-; 6-chloro-; 6-iodo-; 6-triazo-; 6-nitroso-.

*Depressant*: Unsubstituted; 6-amino-; 6-nitro-; 6:7-dimethoxy-.

*Little or No Effect*: 6-amino- hydrochloride; 6-fluoro-; 6-thiocyano-; 6-mercapto-; 6-arsonic acid; 6-stibonic acid; 6-chloromercuri-; 6:7-dihydroxy-bis-(2:3-dihydro-3-ketobenzo-1:4-thiazin-6-yl).

*Phenothiazine Derivatives.*—The effect of phenothiazone, thionol, and phenothiazine sulphoxide on the liver fluke and roundworm has already been described (Mackie and Raeburn, 1952c; Mackie, 1953). Since the 10-aminoacetylphenothiazines possess interesting pharmacological properties (Dahlbom and Ekstrand, 1951), some of these, and a number of other phenothiazine derivatives, were tested against roundworm and liver fluke *in vitro*. The results are recorded in Table II, except for the salts and esters of β-10-phenothiazinylpropionic acid and compounds weakly active or inactive.

The sodium, α-phenylethylammonium, S-benzyl-isothiuronium, and piperazinium salts of β-10-phenothiazinylpropionic acid were paralysant towards the liver fluke at 1:1,000, but only the sodium salt had any depressant action against the roundworm. Of the esters (*normal* from methyl to octyl; *iso*propyl; *iso*-, *sec*.-, and *tert*.-butyl; *p*-nitrobenzyl, and 10-phenothiazinylcarbonylmethyl; formula IV) only the *iso*butyl ester was active against both helminths: it was strongly depressant towards roundworm and depressant towards liver fluke. The latter helminth was strongly depressed by the *iso*propyl ester, but the *sec*.- and *tert*.-butyl esters produced stimulant effects.

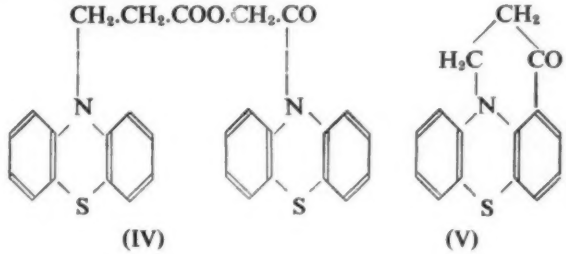
TABLE II

*IN VITRO* EFFECT OF PHENOTHIAZINE DERIVATIVES ON *ASCARIS LUMBRICOIDES* AND *FASCIOLA HEPATICA*

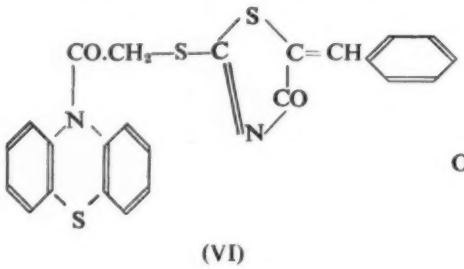
(P = paralysant; L = lethal)

| Compound | Effect On | |
|---|---|---|
| | *Ascaris* | *Fasciola* |
| Lauth's violet .. .. .. .. | − | + |
| Methylene blue .. .. .. | − | L (1:2,000) P (1:3,000) |
| 3:7-Dinitro-10-acetylphenothiazine sulphoxide .. .. .. .. | + | − |
| 10-Dimethylaminoacetylphenothiazine | + | − |
| 10-Ethylaminoacetylphenothiazine .. | + | P (1:2,000) |
| 10-Diethylaminoacetylphenothiazine | + + | P (1:1,000) |
| β-10-Phenothiazinylpropionic acid .. | − | L (1:3,000) |
| γ-10-Phenothiazinyl-γ-ketobutyric acid | − | + + |
| 5′:6′-Dihydro-4′-ketopyridino-(3′:2′:1′-1:10a:10)phenothiazine semicarbazone .. .. .. | + | − |

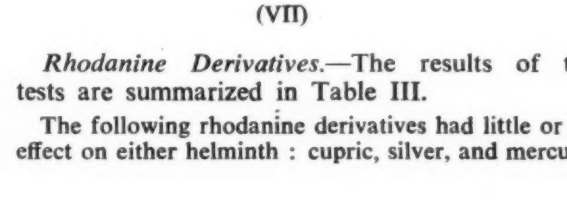
The following phenothiazine derivatives had little or no effect on either helminth: 1:3:7:9(?)-tetrachloro-; 10-methyl-; 3-formyl-10-methyl-; 10-acetyl-; 10-chloroacetyl-; 10-phenylacetyl-; 10-benzoyl-; 10-(2′:4′-dichlorobenzoyl)-; 10-(4′-nitrobenzoyl)-; 10-(3′:5′-dinitrobenzoyl)-; 10-anisoyl; 10-(toluene-*p*-sulphonyl)-; 10-(*p*-acetamidobenzenesulphonyl)-; 10-piperidylacetyl-; 10-morpholinylacetyl-phenothiazines; β-10-phenothiazinylpropionitrile; β-10-phenothiazinylpropion-*p*-toluidide; β-10-phenothiazinylpropion-*p*-bromoanilide; 5′:6′-dihydro-4′-ketopyridino-(3′:2′:1′-1:10a:10)phenothiazine (V), and its benzylidene derivative.




(IV) (V)




(VI)




(VII)

*Rhodanine Derivatives.*—The results of the tests are summarized in Table III.

The following rhodanine derivatives had little or no effect on either helminth: cupric, silver, and mercuric

TABLE III
*IN VITRO* EFFECT OF RHODANINE DERIVATIVES ON *ASCARIS LUMBRICOIDES* AND *FASCIOLA HEPATICA*

| Compound | Effect On | |
|---|---|---|
| | *Ascaris* | *Fasciola* |
| Rhodanine | + | P (1 : 1,000) |
| 3-Allylrhodanine | ++ | P (1 : 1,000) |
| 5-*iso*Nitroso-3-allylrhodanine | P (1 : 1,000) | L (1 : 2,000) |
| | | P (1 : 4,000) |
| Benzylidenerhodanine | — | L (1 : 10,000) |
| Benzylidene-3-allylrhodanine | + | — |
| *o*-Chlorobenzylidenerhodanine | — | L (1 : 2,000) |
| *p*-Chlorobenzylidenerhodanine | — | P (1 : 1,000) |
| *o*-Nitrobenzylidenerhodanine | ++ | L (1 : 2,000) |
| | | P (1 : 3,000) |
| Salicylidenerhodanine | + | L (1 : 16,000) |
| *p*-Hydroxybenzylidenerhodanine | — | L (1 : 1,000) |
| | | P (1 : 4,000) |
| Anisylidenerhodanine | — | ++ |
| Cinnamylidenerhodanine | — | ++ |
| *o*-Nitrocinnamylidenerhodanine | — | ++ |
| Furfurylidenerhodanine | — | L (1 : 3,000) |
| | | P (1 : 4,000) |
| 10-Methylphenothiazine-3-formylidenerhodanine | + | + |
| Quinrhodine | ++ | ++ |

rhodanides; 3-allylrhodanyl-5-*p*-dimethylaminoanil; 4:5′ - dehydro - 4:5′ - bis - 3 - allylrhodanyl - 5 - *p* - dimethylaminoanil; 5:5′ - dehydro - 5:5′ - bis - 3 - allylrhodanine; benzylidenerhodanine 2-phenylhydrazone; piperonylidenerhodanine; *p*-dimethylaminobenzylidenerhodanine; 10-methylphenothiazine-3-formylidene-3-allylrhodanine; S-(10-phenothiazinylcarbonylmethyl)-5′-benzylidenerhodanine (VI); S-(10-phenothiazinylcarbonylmethyl) - 5′ - (*p* - chloro - benzylidene)rhodanine; 2:4-di-(2′:4′-dinitrophenyl)-rhodanine (VII).

*Miscellaneous Compounds.*—A number of miscellaneous compounds, such as aliphatic and aromatic halogen compounds, allyl compounds, mercury compounds, aromatic amines, phenols, pyridines, etc., were tested *in vitro* against both helminths. Some of the compounds had shown promise as sclerostome larvicides (Parnell and Mackie, 1952).

Of the halogenated compounds tested, carbon tetrabromide was the most promising, since it was six times more lethal (1:6,000 against liver fluke) than the tetrachloride. The tetrabromide was paralysant at 1:10,000, and had about the same potency against *Ascaris* (paralysant at 1:2,000) as the tetrachloride. Of the α-, β-, γ-, and δ-isomers of benzene hexachloride only the δ- was active (paralysed liver fluke at 1:12,000).

Some allyl compounds were effective, especially the iodide (roundworm paralysed at 1:5,000; liver fluke killed at 1:5,000) and the *iso*thiocyanate (liver fluke killed at 1:2,000, paralysed at 1:8,000).

Mercuric chloride and two of its organic derivatives were lethal towards liver fluke (mercuric chloride and ethylmercuric chloride at 1:20,000; ethoxyethylmercuric chloride at 1:16,000), but only ethylmercuric chloride was paralysant towards roundworm (1:2,000).

Diphenylamine was lethal towards liver fluke at 1:20,000 and paralysant towards roundworm at 1:1,000. Its derivatives, and other amino-compounds tested, had little or no effect.

*Ortho-* and *p*-nitrophenols were active, especially the *p*-isomer, towards liver fluke (lethal at 1:4,000; paralysant at 1:12,000). The *o*-isomer was more potent towards roundworm (paralysant at 1:3,000).

No lethal effects were observed on liver fluke with pyridine, the picolines, 2:6-lutidine, quinoline and isoquinoline, and only α-picoline was paralysant, whereas all paralysed the anterior preparations of roundworm. Pyridine and α-picoline were the most effective against the latter preparation (cf. Baldwin, 1948).

*pseudo*Thiohydantoin had little effect (cf. rhodanine).

Essential oil from *Artemisia maritima*, containing 65% β-thujone and 16% cineol-1:8, was lethal to liver flukes at 1:2,000 and paralysed at 1:3,000.

2 - Amino - 5 - nitrothiazole (Enheptin - T), a remedy for coccidiosis, was tested against both helminths, but had no effect.

Conessine dihydrochloride had no effect on either helminth. Janot and Cavier (1949) indicated that it might be of value in the treatment of helminthiasis, although they found that it did not kill the anterior preparation of roundworm *in vitro*.

Pumpkin seed extract had little or no effect against liver fluke or roundworm *in vitro*, although the seeds have been used against tapeworm.

Sodium azide was very effective against both roundworm (paralysant at 1:10,000) and liver fluke (paralysant at 1:4,000).

## DISCUSSION

*2 : 3-Dihydro-3-ketobenzo-1 : 4-thiazine Derivatives.*—Increase in the length of the side-chain in position 6 generally decreased the anthelmintic potency towards *Fasciola hepatica* (cf. Mackie and Raeburn, 1952a; Mackie and Cutler, 1952). The chloroacetamido-derivative was exceptional in this respect.

The order of potency of the radicals was Br> Cl>$N_3$, NO>I, $CH_3$>$NO_2$, 6:7-dimethoxy>

$NH_2.HCl$, $ClCH_2CONH$>unsubstituted, F>$NH_2$, $CH_3CONH$, CNS, SH, 6:7-dihydroxy, 6:7-diethoxy. The order of potency of the halogens was different from that obtained with *A. lumbricoides*. This was not surprising, since the two helminths belong to different phyla.

The result with benzo-1:4-thiazine was interesting, since liver fluke is generally more sensitive than roundworm: nevertheless the absence of the $-CH_2-CO-$ group increased the *in vitro* activity against the latter, but decreased the potency against the former.

*Phenothiazine Derivatives.*—The introduction of methyl groups into Lauth's violet conferred lethal properties towards liver fluke (cf. methylene blue, which has been used as an anthelmintic). The presence of a second phenothiazine residue in (IV) does not produce any marked effect.

It was surprising that the presence of the $-CH_2-CO-$ group in (V) and its benzylidene derivative had little or no effect on either helminth (cf. Baldwin, 1948).

*Rhodanine Derivatives.*—The position of the substituent groups in the benzene nucleus, and also any substitution in the 2 and 3 positions in the rhodanine nucleus, had a marked effect on the potency of the corresponding benzylidene-rhodanines.

The presence of a phenothiazine residue in the molecule usually produced little or no effect, and replacement of the $-CH_2-CO-$ group in rhodanine by a quinoline residue (quinrhodine) destroyed the paralysant effect on liver fluke.

Rhodanine had only half the potency of 2:3-dihydro-3-ketobenzo-1:4-thiazine towards liver fluke (Mackie and Raeburn, 1952a), but produced the same effect on roundworm.

*Miscellaneous Compounds.—In vivo* experiments with carbon tetrabromide would be desirable, not only to ascertain its anthelmintic activity, but also to study its effect on the liver, which is damaged by carbon tetrachloride. The tetrabromide had high larvicidal potency (Parnell and Mackie, 1952).

Rico (1927) found that allyl *iso*thiocyanate paralysed *A. lumbricoides in vitro*, but did not give a minimum effective concentration. It has been suggested as an anthelmintic against lungworm (Mathey, 1945). The allyl compounds were very effective larvicides, especially the iodide and *iso*thiocyanate (Parnell and Mackie, 1952). The three mercury compounds tested had similar larvicidal properties, especially ethylmercuric chloride, which merits further investigation.

Although diphenylamine only paralysed roundworm at 1:1,000, Guthrie (1940) had found it to be effective against ascarids in dogs.

The greater potency of pyridine and its derivatives, and of quinoline and of isoquinoline, on roundworm than on liver fluke is exceptional. It may be that these compounds can penetrate the cuticle comparatively easily.

## Summary

1. Derivatives of 2:3-dihydro-3-ketobenzo-1:4-thiazine, phenothiazine and rhodanine, and some miscellaneous compounds, have been tested *in vitro* against *Fasciola hepatica* and the anterior preparation of *Ascaris lumbricoides*.

2. 2:3-Dihydro-3-ketobenzo-1:4-thiazine derivatives showed only depressant effects, when active, towards *A. lumbricoides*, but paralysant effects were observed with some derivatives, particularly the 6-bromo-compound, on liver fluke. Increase in the length of the side-chain usually decreased the anthelmintic potency towards liver fluke.

3. Some of the aminoacetylphenothiazines were active against liver fluke, and $\beta$-10-phenothiazinylpropionic acid was lethal.

4. 5-*iso*Nitroso-3-allylrhodanine was the only rhodanine derivative which paralysed *Ascaris*, but some, especially the benzylidene compounds, were lethal to the liver fluke.

5. Amongst the miscellaneous compounds the following were very active: allyl iodide and sodium azide against *Ascaris*; carbon tetrabromide; benzene hexachloride; allyl iodide and *iso*thiocyanate; mercuric chloride; ethylmercuric chloride; ethoxyethylmercuric chloride; diphenylamine, and *p*-nitrophenol against liver fluke.

We wish to thank the Agricultural Research Council for financial support; Professor Baldwin, who trained one of us (G. M. S.) in his kymographic technique; and Messrs. Norval and Crombie, Edinburgh City Abattoir, for collection of parasites.

We are indebted to Professor R. D. Haworth, F.R.S., for the sample of conessine dihydrochloride, and to T. & H. Smith, Ltd., for the Artemisia oil and cineol-1:8.

The assistance of Miss D. Daniels in the biological testing is gratefully acknowledged. Our thanks are also due to Dr. Parnell for his interest.

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# THE EFFECT OF ANTI-CHOLINESTERASE AGENTS ON THE RAT'S BLOOD PRESSURE

BY

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*From C.D.E.E., Ministry of Supply, Porton, Wilts*

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In most species, the effect of systemic intoxication with an anti-cholinesterase—for example, HETP (hexaethyltetraphosphate), TEPP (tetraethyl pyrophosphate), dyflos (di*iso*propylphosphorofluoridate), Tabun (ethyl *NN*-dimethylphosphoramidocyanidate), or neostigmine—is predominantly the production of a profound fall in blood pressure (Modell, Krop, Hitchcock and Riker, 1946 ; Heymans and Jacob, 1947 ; Lundholm, 1949 ; Burgen, Keele and Slome, 1949 ; Verbeke and Votava, 1949 ; Salerno and Coon, 1949 ; Holmstedt, 1951). We have recently noticed that the intravenous administration of the anti-cholinesterase " Sarin " (*iso*propyl methylphosphonofluoridate) to intact rats produces a sharp rise in blood pressure, then one or two oscillations about the point of increased pressure, followed by a very gradual fall in pressure over the next several minutes (10–180 min. in different animals) back to the pre-injection level (Fig. 1). The cause of this sustained rise in blood pressure produced by sarin has been investigated.

## METHODS

White rats of homogeneous strain and weighing 350–500 g. were used. They were anaesthetized with urethane (1.25 g./kg. subcutaneously), and polythene cannulae were inserted into the carotid artery, to record blood pressure, and into the femoral vein.

With the doses of sarin used respiratory embarrassment or failure may occur and so may interfere with the blood-pressure recording or response. Therefore, in many experiments, even respiratory exchange was maintained throughout by means of a miniature Starling " Ideal " pump. The same pattern of cardiovascular response has, however, been seen in rats that were not sustained by artificial ventilation.

Spinal preparations were made by transecting the spinal cord between C1 and C2 and destroying the brain.

## RESULTS

The hypertensive effect of sarin is best seen following a fairly large dose (40–60 μg./kg.), but is still evident after smaller doses. The effects of rapidly repeated small doses can be summated up to a certain point ; then the general blood-pressure level falls, although each successive sarin dose produces a transient, small rise (Fig. 2). With higher single doses (e.g. 90 μg./kg.) the animal dies before the sustained rise in blood pressure has become established.

Bilateral vagotomy does not affect the results, but if sarin is given to a spinal rat only a relatively slow, small and short-lasting increase in blood pressure is caused (Fig. 3). The failure of sarin to produce a sustained elevation of blood pressure in this experiment is not due to the low blood pressure presented by a spinal rat. Thus, if the blood pressure is lowered, by bleeding or by small doses (10–20 mg./kg.) of C6, sarin still produces a typical hypertensive response. This suggests that the hypertensive action is central in origin. Acetylcholine is probably involved as transmitter at some point, since in the previously atropinized rat (intact

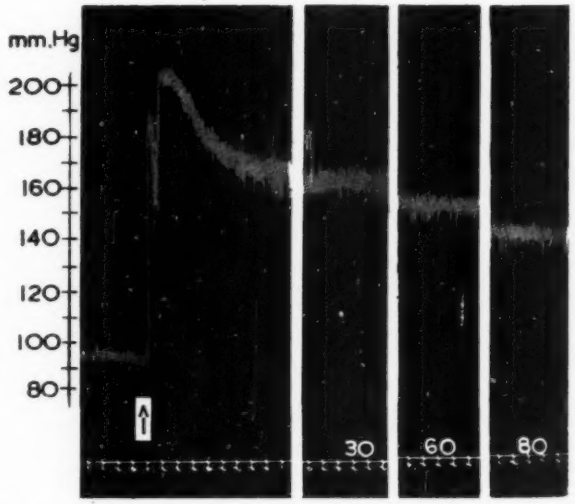


FIG. 1.—Rat 370 g., urethane. Spontaneous respiration. At arrow 40 μg. sarin/kg. i.v. Numerals are times in min. after administration of sarin. (Time scale is 1 min. in all figs.) Shows sustained hypertensive effect of sarin.

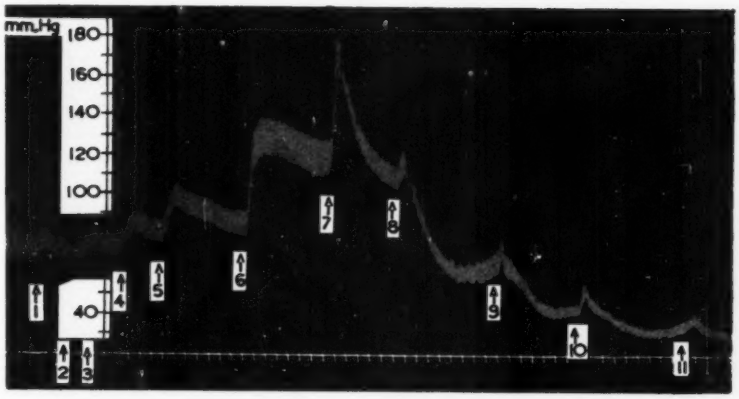


FIG 2.—Rat 400 g., urethane. Artificial ventilation. At each arrow 5 μg. sarin/kg. i.v. (total of 11 doses). Shows effect of repeated small doses of sarin.

or spinal) sarin produces again only a slow and minor rise in blood pressure (Fig. 4). Similarly, if successive doses of atropine are administered after sarin, a step-like depression of the elevated blood pressure is produced.

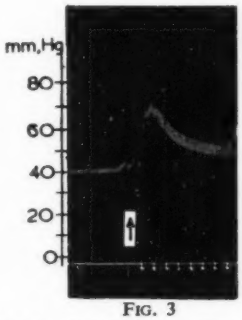


FIG. 3

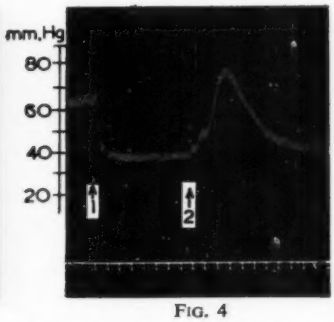


FIG. 4

FIG. 3.—Rat 470 g., urethane. Spine transected, brain pithed. At arrow 40 μg. sarin/kg. i.v. Shows small blood-pressure rise in spinal rat.

FIG. 4.—Rat 310 g., urethane. Artificial ventilation. At 1st arrow 10 mg. atropine/kg.; at 2nd arrow 40 μg. sarin/kg. i.v. Shows small blood-pressure rise in atropinized rat.

If C6 (10–20 mg./kg.) is given to a rat during the sustained pressor response following sarin, the blood pressure is immediately reduced (Fig. 5). Pre-treatment with large doses of C6 (200–400 mg./kg.) prevents the usual pressor response to sarin.

The central stimulant action of sarin is effected, therefore, via the sympathetic nervous system. Liberation of adrenaline from the adrenal glands would not seem to be important, since sarin still raises the blood pressure of adrenalectomized rats, and this rise can be inhibited by small doses of C6.

A sympathetic, peripheral vascular mechanism is probably involved, since peripheral blockade of sympathetic impulses with ergotamine or tolazoline alters the response. If either of these substances is administered before sarin, the latter produces only a preliminary sharp rise, but no sustained elevation, of blood pressure (Fig. 6). If sarin is given first, and then tolazoline during the period of raised blood pressure, the latter is at once temporarily reduced.

The sustained rise in blood pressure induced by sarin is probably due mainly to constriction of the skin arterioles: in the skinned rat only an immediate temporary rise in pressure, with no continued elevation, is seen (Fig. 7). In conformity with this, sarin produces a maintained pressure rise when administered to an eviscerated rat. This rise is not as long-lasting as that occurring in the intact rat, so that some arteriolar constriction may occur in the splanchnic area.

That a direct central action is involved is also suggested by the observation that a small dose (5 μg.) of sarin, injected into the fourth ventricle, causes a similar sustained rise in blood pressure (Fig. 8). An injection of acetylcholine into the same site produces a fall in blood pressure. This dissimilarity in the actions of acetylcholine and sarin may be owing to failure of the former to

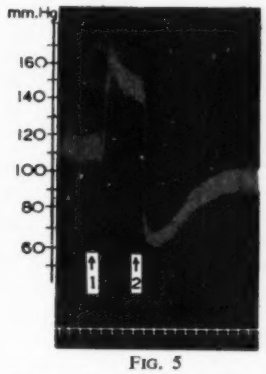


FIG. 5

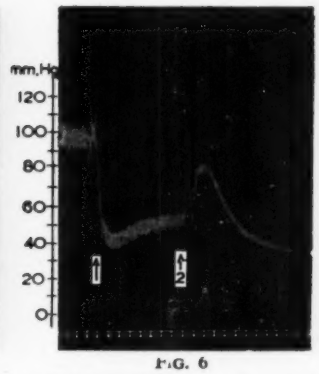


FIG. 6

FIG. 5.—Rat 390 g., urethane. Artificial ventilation. At 1st arrow 40 μg. sarin/kg. At 2nd arrow 10 mg. C6/kg. i.v. Shows that a small dose of C6 after sarin reduces blood pressure.

FIG. 6.—Rat 390 g., urethane. Artificial ventilation. At 1st arrow 10 mg. tolazoline/kg. At 2nd arrow 40 μg. sarin/kg. Shows small pressor effect after treatment with tolazoline.

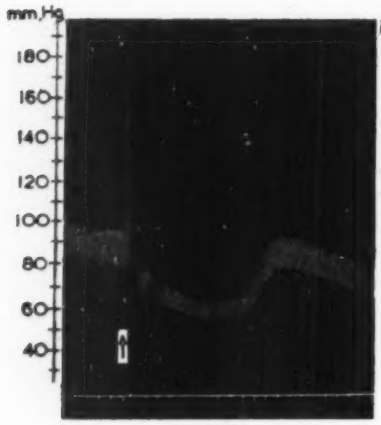


FIG. 7

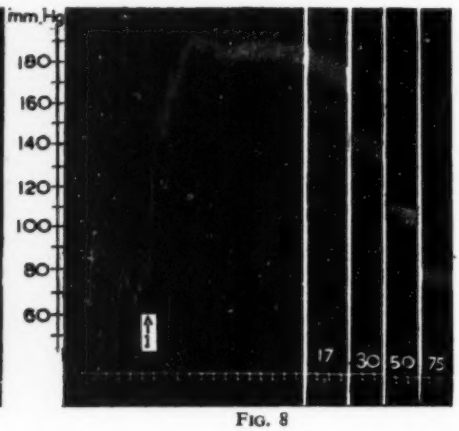


FIG. 8

FIG. 7.—Rat 480 g., urethane. Artificial ventilation. All skin removed (head, feet, and scrotum not skinned). Body placed in saline bath at 37°. At arrow 40 μg. sarin/kg. intravenously. There is no sustained pressor effect in skinned animal.

FIG. 8.—Rat 420 g., urethane. Artificial ventilation. At arrow 5 μg. sarin/rat through atlanto-occipital membrane. Shows effect of small dose of sarin injected to 4th ventricle. Numerals indicate time (min.) after sarin.

penetrate into the brain substance, although some leakage into the systemic circulation occurs. The direct central action of sarin can still be prevented by systemic atropinization.

Another indication of the enhanced sympathetic tone in these rats is that the heart rate (measured from the e.c.g.) is increased during the period of hypertension—in a typical instance the heart rate before sarin (40 μg./kg.) was 270/min.; during the phase of rising blood pressure it was 390/min.; and at the peak of the blood-pressure rise it reached 450/min.

In other species, where a fall of blood pressure is produced by sarin, this is accompanied by a slowing of the heart (Holmstedt, 1951).

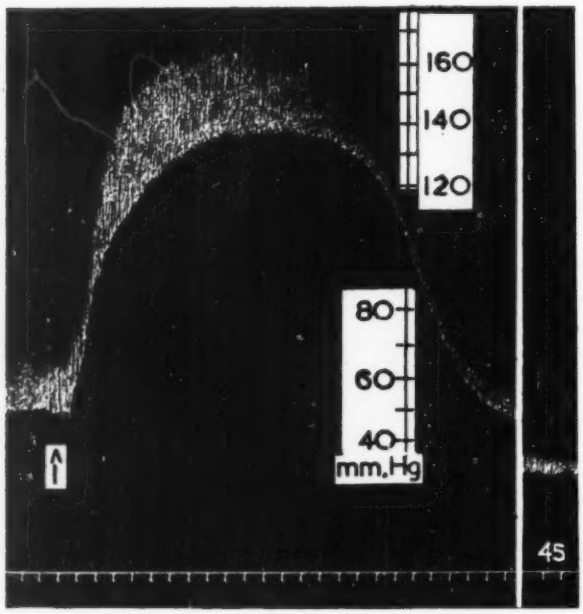


FIG. 9.—Rat 470 g., urethane. Artificial ventilation. At arrow 1 mg. dyflos/kg. intravenously. Shows hypertensive effect of dyflos in ther at.

The hypertensive action of sarin in the rat is not peculiar to that compound, but is shared by other anti-cholinesterases, such as dyflos (Fig. 9), eserine, TEPP, and E.600. Atropine and C6 affect the response to these agents in the manner described for sarin.

## DISCUSSION

From the above results we can conclude that sarin, when given intravenously to the rat, produces a rise in blood pressure which is due to a central action, possibly on the vasomotor centre. The effect is not seen in the spinal rat or following atropine. The latter observation suggests that the central phenomenon involves cholinergic transmission.

The central stimulation presumably acts through the sympathetic nervous system, and this sympathetic "drive" can be blocked at the ganglia by hexamethonium bromide and, more peripherally, by ergotamine or tolazoline. It would appear that the vessels of the rat's skin are those chiefly concerned in this sympathetic action.

Other anti-cholinesterases can produce a similar hypertension in the rat, so that the phenomenon presumably has the inhibition of cholinesterase as its basis. The rise in blood pressure is, further, not peculiar to the intravenous route of administration, since it has also been seen after the intraventricular or the intracarotid injection of sarin.

In other species anti-cholinesterases, when administered intravenously, cause a marked lowering of the blood pressure, although a transient rise of pressure is often seen before the profound fall takes place (Holmstedt, 1951). This fall in blood pressure is accompanied by a marked slowing of the heart and by dilatation of the small vessels in the limb muscles; whereas, if sarin is injected in small doses into the vertebral artery or

the cisterna magna of a dog, a rise in blood pressure is the invariable response (Wilson, personal communication). Therefore a central hypertensive action can be seen in another species than the rat. In the dog following intravenous administration, it must be presumed that the peripheral effects on the cardiovascular system predominate over the central hypertensive action.

These varying responses in the different species suggest that there may be corresponding differences in the nature and the sensitivity of the receptor substances and the cholinesterases of the tissues of these species. This is being further investigated.

## Summary

1. Sarin, dyflos, eserine, TEPP, and E.600 all produce hypertension when administered to rats in near-lethal doses.

2. This hypertension is apparently effected by a central mechanism acting through the sympathetic nervous system on the blood vessels of the skin.

We are indebted to the Chief Scientist, Ministry of Supply, for permission to publish these results.

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# STUDIES ON THE TOXICITY OF ALKYL TIN COMPOUNDS

BY

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In recent years the toxicity of tin has aroused little interest, but in the latter part of the last century and the first decade of this, considerable attention was paid to the possible contamination of food preserved in cans (White, 1881 ; Ungar and Bödlander, 1886 ; Lehmann, 1902 ; Eckhardt, 1909 ; Schryver, 1909). It was concluded that there was no serious risk ; since then there have only been occasional studies on the toxicity of tin compounds (Salant, Rieger, and Trenthardt, 1914 ; Handovsky, 1925 ; Schwartzer and Clarke, 1927 ; Seifter and Rambousek, 1943). A suggestion that the fungicidal properties of certain organic tin compounds might have industrial application (Van der Kerk and Luijten, 1954) coupled with their use as " plasticizers " has rekindled interest in this subject. In most reference books the toxicity of tin tends to be discounted, but preliminary work with its alkyl derivatives showed that on a molar basis tin was five times as toxic as beryllium. Its potentialities have been underestimated because of the difficulty of introducing it into the body in a soluble form. The full toxicity is only seen when the alkyl derivatives are used, some of which are volatile and soluble in water at *p*H 7. A number of these and related compounds have now been examined. Many of their toxic properties are already well outlined (White, 1881 ; Ungar and Bödlander, 1886), but little attention has been paid to them recently (Seifter, 1939a ; McCombie and Saunders, 1947) and it is now possible to extend these findings. Later knowledge, particularly regarding their action on the central nervous system, has an important bearing on the assessment of the possible hazards to health during the production and use of the alkyl tin compounds.

## METHODS

Experiments were carried out on albino rats fed on M.R.C. diet 41 (Bruce and Parkes, 1949) ; on rabbits, guinea-pigs, and domestic fowls fed on M.R.C. diet 18 (Bruce, 1947), and on cats. Cabbage was added to the diet of the guinea-pigs. The tin compounds used, with their chemical properties, sources, and method of administration, are shown in Table I.

The cardiovascular effects of these compounds were observed in the cat and rat under pentobarbitone sodium anaesthesia (50 mg./kg. body wt. i.p.) and in the rabbit under urethane (4.0–5.0 ml. 25% w/v solution/kg. i.v.). The blood pressure was recorded from the carotid artery with a mercury manometer. Rectal and skin temperatures were determined with a thermocouple.

TABLE I

TIN COMPOUNDS USED, WITH THEIR VEHICLES AND ROUTES OF ADMINISTRATION

| Compound | Vehicle | Route |
|---|---|---|
| *Tetraethyl tin | Nil | i.v., i.p. |
| *Trimethyl tin sulphate | 0·5% (w/v) in 0·9% (w/v) NaCl, *p*H 7·4 | i.p., i.v. |
| *Triethyl tin sulphate | 0·5% in 0·9% NaCl, *p*H 7·4 | i.p., i.v., p.o. |
| * " " " | 1% in arachis oil | p.o. |
| * " " " | 6% in dimethyl phthalate | p.c. |
| *Triethyl tin hydroxide | 0·5% in arachis oil | p.o. |
| *Tri-*n*-propyl tin acetate | 1% " " " | " |
| *Tri-*iso*-propyl tin acetate | 1% " " " | " |
| Tri-*n*-butyl tin acetate | 2% " " " | " |
| " " " | 0·5%, 2% in Tween 80 | i.p. |
| *Tri-*n*-hexyl tin acetate | 2% in arachis oil | p.o. |
| *Triphenyl tin acetate | 2·5% and 4% in arachis oil | p.o., i.p. |
| *Diethyl phenyl tin acetate | 2·5% in arachis oil | p.o. |
| *Diethyl tin dichloride | 2% in Tween 80 | i.p. |
| * " " " | 2% in arachis oil | p.o. |
| * " " " | 2% in propylene glycol | i.v. |
| *Diethyl tin diiodide | 2% in Tween 80 | i.p. |
| * " " " | 2% in arachis oil | p.o. |
| *Dibutyl tin dichloride | 1% " " " | " |
| *Dibutyl tin dilaurate | Suspension in arachis oil | " |
| *Monoethyl tin trichloride | 2% in water neut. *p*H 5–6 | i.v., i.p. |
| Stannic chloride (B.D.H.) | 0·4% in arachis oil | i.p. |
| Stannic sodium chloride (B.D.H.) | 0·7% in water *p*H 2·3 | i.v. |
| †Stannic lactate | 1·3% " " *p*H 7·0 | " |
| ‡Stannic citrate | 4% " " *p*H 7·0 | " |
| §Sodium stannitartrate (34·8% Sn) | 8% " " *p*H 7·0 | " |
| ‖ Sodium stannitartrate (17·4% Sn) | 8% " " *p*H 7·0 | " |
| §Sodium stannous tartrate (19% Sn) | 1% " " *p*H 7·0 | " |

* Prepared by Professor Van der Kerk, Utrecht, and supplied by the Tin Research Institute.

† Prepared by Dr. W. N. Aldridge.

‡ Prepared by the method of Seifter and Rambousek (1943).

§ Prepared by Dr. J. W. Price, Tin Research Institute.

‖ Prepared by Dr. D. F. Heath.

p.o., by mouth; i.p., intraperitoneal; i.v., intravenous; p.c., percutaneous.

Neuromuscular conduction was studied in rabbits under urethane and in a hen under pentobarbitone anaesthesia. The exposed sciatic nerve was stimulated by supramaximal rectangular impulses of 200 μsec. duration, and the contractions of the gastrocnemius or tibialis anterior were recorded with a Brown-Schuster myograph. The rat phrenic nerve-diaphragm preparation (Bülbring, 1946) was also used, as modified by Barnes and Duff (1953). The experiments on frogs were done during March, and the contractions of the isolated rectus muscle, suspended in frog-Ringer's solution, were recorded with an isotonic lever.

Blood sugar was determined by the method of Haslewood and Stookman, and blood non-protein nitrogen with Nessler's reagent, both as described by King (1951). The blood haemoglobin was determined by Haldane's method. The techniques for the estimation of the muscle phosphates have been described by Stoner, Threlfall, and Green (1952). Cholinesterase activities were determined as described by Davison (1953). Tin was determined polarographically (Godar and Alexander, 1946) using hydroxylamine HCl to reduce ferric iron.

Some of the rats had their adrenals medullectomized by the method of Evans (1936) as described in Farris and Griffith (1942). The completeness of the medullectomy was checked histologically at the end of the experiment. Intravenous injections in unanaesthetized rats were given by the technique of Ginsburg and Heller (1953).

The histology of the tissues was studied on material fixed in 10% (v/v) formol-saline or Helly's fluid. The techniques used for the central nervous system were those of Barnes and Denz (1953).

## Results

### *Acute Toxicity of Alkyl Tin Compounds*

*Rat.*—Triethyl tin sulphate was equally lethal on intravenous, intraperitoneal, and oral administration. Intravenously, rats tolerated 5 mg./kg. body weight, but 10 mg./kg. was fatal in 4 days and higher doses caused immediate death. When groups of 5 rats were given triethyl tin sulphate intraperitoneally in doses of 2, 4, 8 and 16 mg./kg. the mortality after a week was 0, 0, 5, 5, giving an LD50 of 5.7 mg./kg. calculated by the method of Weil (1952). A certainly fatal dose was 10 mg./kg. intraperitoneally which caused death usually within 5 days. Higher doses by this route shortened the survival time, so that 40 mg./kg. was fatal in 2 hr. This compound was less effective when given subcutaneously; when a single dose of 25 mg./kg. was applied, in dimethyl phthalate, to the epilated skin of 4 rats, only 1 died although all became ill.

After the intraperitoneal injection of 10 mg./kg. triethyl tin sulphate striking changes appeared within 30 min. The main effect was a generalized weakness, first manifest in a dragging of the hind limbs. This persisted for 3–4 hr., when the rat recovered slightly. However, by the next day the weakness was more marked and generalized and progressed until the rat died, usually on the 3rd day after the injection. The respiratory rate was slightly depressed and there was vasodilatation in the ears. This latter was very marked after 20 mg./kg. The intensity of these changes was rather less in fed than in fasted animals. Although very ill, the rats continued to attempt to eat and drink until they died. The desire for water seemed increased shortly after the injection, but when weakness became profound the intake of food and water was greatly reduced. An inconstant effect was the secretion of "red tears."

Changing the dose or route of injection did not alter this picture. Larger doses accentuated the weakness so that rats were unable to right themselves 20 min. after the intraperitoneal injection of 30–40 mg./kg. The effects of 10 mg./kg. intraperitoneally could be exaggerated by raising the environmental temperature to 30° C.; the animals quickly became prostrate, and "red tears" were more constant, but the survival time was not shortened.

Trimethyl tin was less toxic. An intraperitoneal dose of 8.8 mg./kg. trimethyl tin sulphate, equivalent in Sn content to 10 mg./kg. triethyl tin sulphate, had no immediate effect. Twenty-four hours later the rats were very excitable, standing up in pairs facing each other as if sparring. This went on almost continuously for a further 24 hr., after which they gradually returned to normal behaviour. Only 1 of a group of 4 died after this dose. With twice this dose of trimethyl tin there was difficulty in moving the hind limbs, and after 24 hr. they exhibited almost continuous, generalized tremors. "Red tears" were seen at this time and death occurred in 2–3 days. The certainly fatal oral dose of this compound was 30 mg./kg.

Various other alkyl tin compounds were examined and their commonly fatal doses, or, for the less toxic members, the highest dose given, are shown in Table II. The occasional difference between the toxicity of oral and intraperitoneal doses suggests that compounds other than the trimethyl and triethyl are poorly absorbed from the gut. There was also a marked difference between the diethyl and triethyl compounds. Although, on intraperitoneal injection, the former was not much less toxic than the latter, the certainly fatal dose being 15 mg./kg., it did not produce any of the characteristic effects of the triethyl tin. After diethyl tin the rats merely became ill, and eventually died without showing

TABLE II

THE CERTAINLY LETHAL DOSES (MG./KG. BODY WEIGHT) OF A RANGE OF ORGANIC TIN COMPOUNDS

| Compound | Rabbit | | Rat | | Guinea-pig |
|---|---|---|---|---|---|
| | Oral | i.p. | Oral | i.p. | Oral |
| Trimethyl .. .. | — | — | 30 | 16 | — |
| Triethyl .. .. | 10 | 10 | 10 | 10 (LD50 5·7) | 5–10 |
| Tri-*n*-propyl .. | — | — | >40 | — | — |
| Tri-*iso*-propyl .. | — | — | 80 | — | — |
| Tri-*n*-butyl .. | 60 | — | 50–100 | 10 | 20 |
| Tri-*n*-hexyl .. | — | — | >100 | — | — |
| Triphenyl .. .. | >40 | — | >150 | — | 10 |
| Diethyl phenyl .. | — | — | 50–100 | — | — |
| ,, dichloride | — | — | >40 | 15 | — |
| ,, diiodide .. | — | — | 100 | 26 | — |
| Dibutyl dichloride | — | — | 100 | — | — |
| ,, dilaurate .. | — | — | — | 85 | — |
| Monoethyl trichloride | — | — | — | 200 | — |

any distinctive changes. The two compounds also differed in their response to dimercaprol (BAL). Premedication and continued therapy with dimercaprol (30 mg./kg. intramuscularly twice a day) had no effect on the response to triethyl tin, but it antagonized the effects of diethyl tin (Table III). When tested in equimolecular amounts the toxicity of diethyl tin dichloride and diethyl tin diiodide was the same. Diethyl tin was equally toxic to the mouse and rat.

Monoethyl tin trichloride, given intraperitoneally (200 mg./kg.), was much less toxic than the other compounds and produced no distinctive symptoms.

TABLE III

EFFECT OF DIMERCAPROL (BAL) (30 MG./KG. I.M. IN OIL) ON THE SURVIVAL TIME OF RATS GIVEN 20 MG./KG. DIETHYL TIN DICHLORIDE (I.P. IN TWEEN 80)

Untreated rats were given equivalent volume of arachis oil i.m. Number of rats shown in parentheses

| Treatment | Survival Time | |
|---|---|---|
| | Treated | Untreated |
| BAL given 30 min. before and 6 hr. after injection of diethyl tin | 3 days (1)<br>7 ,, (2)<br>>7 ,, (1) | 5¼ hr. (1)<br><24 ,, (3) |
| BAL given at 2 and 6 hr. after injection of diethyl tin | >7 ,, (3) | <24 ,, (3) |

*Rabbit.*—The rabbit was more sensitive to triethyl tin sulphate than the rat, so although 1.0 mg./kg. intravenously had little effect beyond causing a few running movements at the time of the injection and some vasodilatation in the ears, 3.0 mg./kg. was fatal within 24 hr. After this dose the initial struggling was more severe and respirations were increased in depth. The limbs quickly became flaccid (hind>fore) and there was difficulty in righting for about 15 min. At 5.0 mg./kg. there was a greater initial increase in the rate and depth of respiration, often amounting to stridor, sometimes with squealing and rubbing of the nose. Complete flaccid prostration quickly followed and lasted about an hour. During this period breathing became shallow and rather rapid. The ear vessels were dilated, the pupils were contracted, and nystagmus was sometimes seen. The corneal reflex was sluggish but never absent. After this period of prostration the rabbit usually recovered sufficiently to move about the cage. Some time (1–5 hr.) later, depending on the dose, involuntary tremors appeared and became progressively worse until within 1–2 hr. the rabbit was having continuous violent convulsions. These persisted for an hour or so until the rabbit finally collapsed and died. After 10 mg./kg. intravenously the rabbit usually passed directly from prostration to convulsions and death within 3 hr. Death sometimes occurred while the animal was prostrate. The intraperitoneal or oral administration of 10 mg./kg. triethyl tin sulphate produced the same sequence of events as 5.0 mg./kg. intravenously. Continuous tremors could also be produced by applying it to the skin.

Trimethyl tin was again less effective. An intravenous dose of trimethyl tin sulphate equivalent in Sn content to 5.0 mg./kg. triethyl tin sulphate had no immediate effect other than transitory contraction of the pupil. However, 24 hr. later the rabbit showed the same type of convulsive movements as after triethyl tin although they were less severe. Muscular weakness also occurred after twice this dose of trimethyl tin, but the survival time was longer than after the triethyl compound. The effects of the tributyl and triphenyl compounds are shown in Table II.

After tetraethyl tin (25 mg./kg. i.v.) the only immediate effects were a slight increase in respiratory rate and vasodilatation in the ears. However, 1.5–2.0 hr. later muscular weakness and prostration appeared and then the sequence of events was as after triethyl tin and the mode of death the same.

The intravenous lethal dose of diethyl tin dichloride was 10 mg./kg. The only immediate effect was a short-lived contraction of the pupil and excessive salivation. Death occurred without any of the characteristic signs seen after triethyl tin.

Monoethyl tin trichloride was much less active than the other alkyl tin compounds. Even 150 mg./kg. i.v. was not fatal, but 70–150 mg./kg. by this route produced certain short-lived effects. Almost immediately after the injection there was hyperpnoea, vasodilatation in the ears, some struggling followed by prostration, muscular

tremors and head drop. Within an hour the rabbit had largely recovered and moved about normally.

*Domestic Fowl.*—In the adult hen 3.0 mg./kg. triethyl tin sulphate intravenously caused immediate collapse with salivation and a few convulsive movements leading to death in a few minutes. After 2.0 mg./kg. there was again immediate prostration with flaccidity of the muscles. The eyes closed and there was excessive salivation. Respiration, after an initial increase in depth, became slow and shallow. The bird was unable to right itself and remained lying on the floor of the cage, apparently unconscious, for 1½–2 hr. It then gradually recovered and on the following day was apparently normal. Prolonged effects were not produced. The birds either died shortly after the injection or recovered completely within a few hours.

Trimethyl tin was about as toxic as the triethyl compound, but the survival time was longer and the immediate effects, though similar, were less severe. After a dose equivalent to 3.0 mg./kg. triethyl tin sulphate the initial effect lasted about ½ hr., after which, although fully conscious, the hen was unable to stand or hold its head up. This state continued until death occurred within 24 hr., preceded by tremors and convulsions as in the rabbit.

In male chicks (about 100 g. body weight) similar responses were observed to these doses of triethyl and trimethyl tin, although there was very little immediate effect after fatal doses of the latter.

*Guinea-pig.*—Systematic tests were not done on this species, but a sufficient number of oral doses were given to show that, whereas the fatal dose of triethyl tin was of the same order as in the rat and rabbit, the guinea-pig was more sensitive to the oral administration of tributyl and triphenyl tin (Table II). Triethyl tin could also be absorbed through the skin of the guinea-pig.

### *Chronic Toxicity of Alkyl Tin Compounds*

*Rat.*—On a diet containing 20 parts per million triethyl tin hydroxide the rats ceased to gain weight (Fig. 1). Paired feeding showed that this was owing to decreased food intake. After 7 days, weakness of the hind legs appeared. This progressed until the rat lay on its side on the floor of the cage. It was cold and breathed slowly. The feet were pink, and, although the hind limbs appeared to be "paralysed," they were withdrawn if the foot was pinched. Even in this condition the rats would still attempt to eat, although the intake of food and water was greatly reduced. These changes reached a maximum in 3–4 weeks, when some of the rats died. This picture was not altered by giving additional aneurine hydrochloride (5 μg./100 g. body weight i.p. daily). If they were returned to the normal diet at this stage signs of poisoning disappeared in about 7 days and in 4 weeks they had reached the body weight of the controls (Fig. 1). About half of those kept on the diet containing triethyl tin hydroxide died at this stage, but the remainder appeared to become resistant to the drug, regaining weight and muscular power (Fig. 1). This recovery process was not altered by increasing the concentration of triethyl tin to 40 parts per million, but at double this concentration they lost weight (Fig. 1) and developed generalized muscular tremors like those of acute trimethyl tin poisoning. This particular group were then killed for histological examination, but these tremors were also recorded in another slightly different experiment. Here the rats were fed 20 parts per million of triethyl tin hydroxide for 24 days, to produce severe poisoning, and were then returned to the normal diet. Eight days later, when they had largely recovered, the triethyl tin diet was resumed and severe tremors appeared after a further 7 days. Although the level of the triethyl tin in the diet was maintained, the intensity of the tremors gradually diminished.

*Rabbit.*—Rabbits tolerated 20 parts per million of triethyl tin hydroxide in the diet, but at 40 and

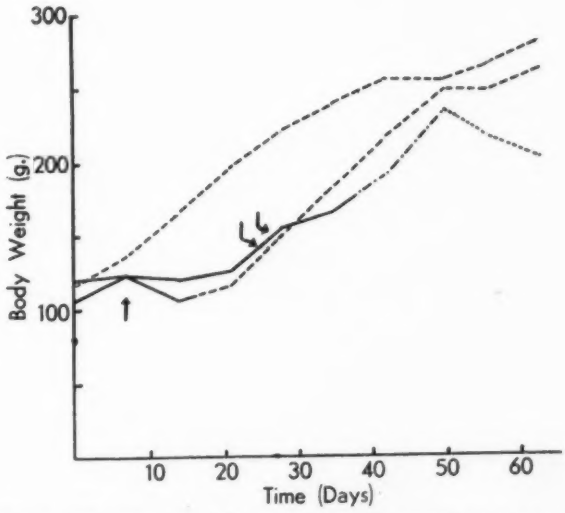


FIG. 1.—The effect of feeding triethyl tin hydroxide on the body weight of rats. Each line shows the mean body weight in a group of 5 rats. First arrow indicates the onset of muscular weakness in the treated groups; other arrows indicate death. - - - - - - - Control diet 41. ——— Control diet 41 + 20 parts per million triethyl tin hydroxide. —.—.—.— Control diet 41 + 40 parts per million triethyl tin hydroxide. . . . . Control diet 41 + 80 parts per million triethyl tin hydroxide.

80 parts per million they developed progressive muscular weakness and loss of weight. The hind limbs were affected first and the rabbits sat hunched up in a characteristic posture. The weakness extended to the fore limbs and, in the end, the rabbit lay sprawled out on the floor of the cage. The limbs were markedly wasted. At the 80 parts per million level, muscular weakness could be produced in eight days without any decline in food intake. These diets were ultimately fatal, but there was considerable variation in both onset of signs and survival time. No tremors or convulsions were seen in these rabbits, nor was there any change in haemoglobin concentration.

*Domestic Fowl.*—Hens tolerated triethyl tin in their diet much better. Even after 15 weeks on a diet containing up to 160 parts per million no ill effects were observed in three hens except for loss of weight due to their dislike of the powdered diet. Because of this the effect of direct oral administration was observed in one hen, and 2.5 mg./kg. triethyl tin hydroxide in arachis oil was given six times a week for four weeks. This had no effect and so the dose was changed to 5.0 mg./kg. triethyl tin sulphate in 0.9% NaCl. After a further month the hen lost weight and developed some muscular weakness.

### *Toxicity of Other Tin Compounds*

Comparison between the behaviour of these alkyl derivatives and that of other tin compounds was difficult. Neutral aqueous solutions of inorganic tin salts cannot be prepared. Stannic chloride in arachis oil was given intraperitoneally to rats without effect, but as a white precipitate was later found in the peritoneal cavity it is doubtful if any was absorbed. Stannic sodium chloride, soluble in aqueous solution at *p*H 3.0, was injected slowly intravenously in rabbits so as to give the amount of tin in effective doses of triethyl tin. The only effect observed was severe hyperpnoea. Of the organic compounds, equivalent doses of stannic lactate were without effect. Stannic citrate was also tried, but, as found by Seifter and Rambousek (1943), it owed its toxicity in acute experiments to its citrate content. More success was obtained with the double salts of tin with sodium tartrate. Three such preparations were used, all proving toxic in the rabbit. Seven daily intravenous doses of 200 mg./kg. of a preparation of sodium stannitartrate containing 33% Sn produced weakness of the hind limbs, loss of weight, and death five days after the last dose. Another preparation containing 17.4% Sn caused death after a single dose of 580 mg./kg. given intravenously. Daily subcutaneous doses, 145 mg./kg., of this compound caused death after 12 days. This compound produced tremors and convulsions similar to those seen after triethyl tin. Sodium stannous tartrate (19% Sn) was also active, and a dose of 132 mg./kg. intravenously, repeated after 24 hr., proved fatal. The effects of all three compounds were delayed in onset, and it was the late effects of triethyl tin which were simulated.

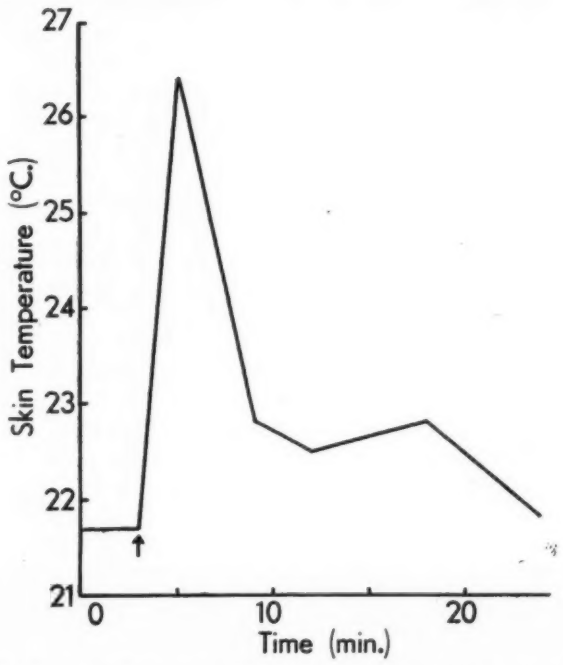


FIG. 2.—The effect, on the skin temperature of the opposite ear, of injecting 10 mg./kg. body wt. triethyl tin sulphate into the ear vein of a rabbit. Time of injection shown by arrow.

### *Pharmacological Actions of Alkyl Tin Compounds*

*Cardiovascular System.*—Vasodilatation in the ears was noted above. The response in the rabbit, as reflected in the skin temperature of the ear, is shown in Fig. 2. Rectal temperature was depressed in both rat and rabbit. On the day following the intraperitoneal injection of 10 mg./kg. triethyl tin sulphate in the rat this temperature was about 34° C., and fell slowly to 28–30° C., when the animal died. After 20 mg./kg. the rectal temperature fell more rapidly—8–10° C. during the first 5 hr. after the injection. In the rabbit, after 5.0 mg./kg. triethyl tin sulphate, the rectal temperature rapidly fell about 3° C.; it remained at the low level until the tremors appeared, when it rose. The fall in rectal temperature probably resulted from increased heat loss with compensatory vasoconstriction (Grayson,

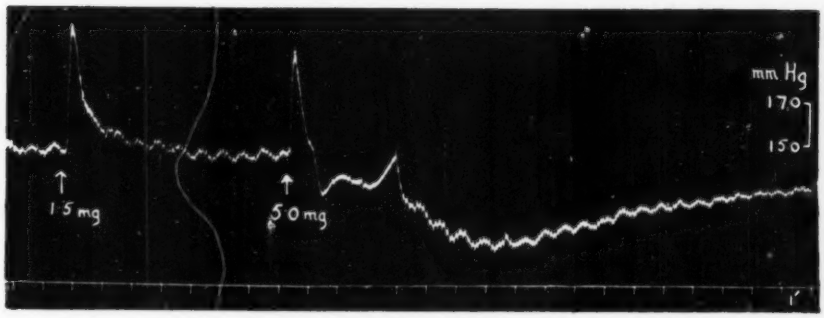


FIG. 3.—The effect of the intravenous injection of triethyl tin sulphate on the blood pressure of a cat (2.2 kg.) under sodium pentobarbitone anaesthesia.

1951). However, recent observations (unpublished) on the effect of triethyl tin on the temperature of the liver and brain suggest that this may not be the whole explanation.

Small intravenous doses (0.12–0.57 mg./kg.) of triethyl tin sulphate raised the blood pressure in the cat. With repeated doses of this order some tachyphylaxis was seen. After larger doses (2.5 mg./kg.) a secondary depressor effect was observed (Fig. 3), becoming more marked as the dose was further increased. Neither the rise nor the fall in blood pressure was significantly altered by atropine sulphate (1.0 mg./kg. i.v.) or splanchnic nerve section. The responses in the rat were rather variable. Most commonly there was a fall in pressure with 0.25 mg./kg. i.v. In the rabbit depressor responses were seen after the intravenous injection of 3.0 mg./kg. triethyl tin sulphate. Trimethyl tin was less active as a depressor agent in this species.

*Neuromuscular Conduction.*—In the rabbit intravenous doses of triethyl tin sulphate up to 3.0 mg./kg. affected the response of the tibialis anterior and gastrocnemius to fast, but not slow, rates of stimulation through the nerve. The response to 500 stimuli/sec. was first affected so that the muscle was unable to hold the tetanus (Fig. 4). This effect was maximal about 5 min. after the injection, and recovery occurred after about 60 min. even after doses within the lethal range. Responses to rates of stimulation below 200/sec. were not affected by the doses used. These changes could not be correlated with those in the blood pressure, which had returned to the pre-injection level at the time of the maximum effect. Trimethyl tin sulphate had a similar, though less pronounced, action.

In contrast, normal responses were obtained to rates of stimulation up to and including 500/sec. in rabbits fed triethyl tin hydroxide (80 parts per

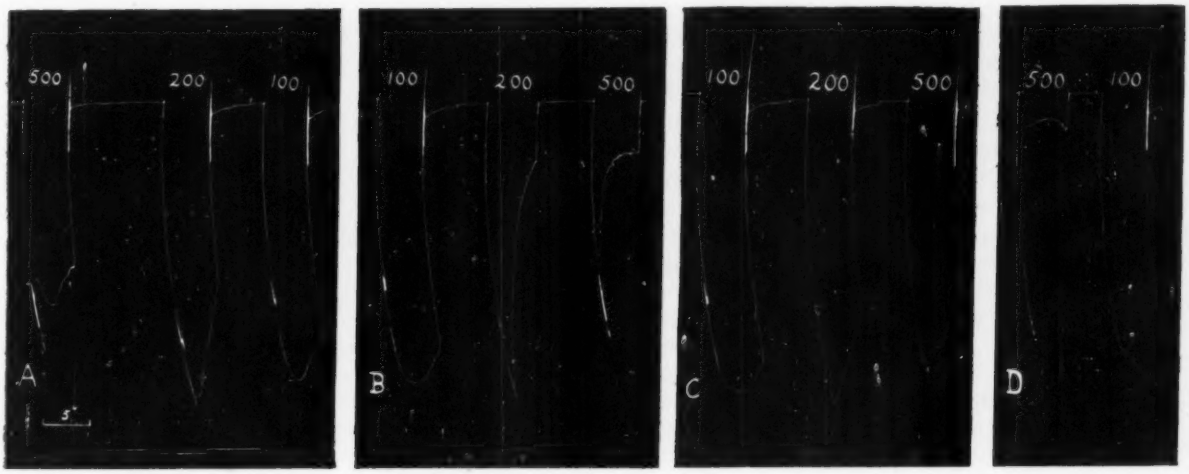


FIG. 4.—The effect of triethyl tin on the response of the tibialis anterior muscle to stimulation of the sciatic nerve in a rabbit (2.5 kg.) under urethane anaesthesia. A, Before administration of triethyl tin; B, 6 min. after 3.0 mg./kg. body wt. triethyl tin sulphate i.v.; C, one hr. later; D, 17 min. after a further dose of 3.0 mg./kg. triethyl tin sulphate. The period of stimulation is shown by the lower tracing; the frequency of stimulation (stim./sec.) is indicated by the numeral above each response.

million) and showing severe weakness of the hind legs. Normal gastrocnemius responses were also found in an isolated experiment on a hen after the intravenous injection of up to 3.0 mg./kg. triethyl tin sulphate.

*Rat Phrenic Nerve-Diaphragm.*—Triethyl tin ($1 \times 10^{-5}$ M) led to a progressive failure of the diaphragm response to single stimuli. Failure to respond became almost complete within 1 hr. and was often associated with some degree of contracture. On removing the tin some recovery took place. At $5 \times 10^{-5}$ M complete failure and a marked, irreversible contracture developed rapidly. At $2.5 \times 10^{-6}$ M there was no change in the response to single stimuli for at least 2 hr., but the preparation by then would no longer hold a tetanus to 500 stimuli/sec. applied for 5 sec. At $1 \times 10^{-5}$ M there was a failure to hold a tetanus to 50 stimuli/sec. In neither case was recovery complete after removal of the triethyl tin.

With trimethyl tin, on the other hand, the effects were rapidly reversed after removing the tin. A concentration of $1 \times 10^{-4}$ M was required to cause a failure to hold a tetanus to 50 stimuli/sec., but this concentration had only a slight effect on the size of the response to single stimuli.

The response of the diaphragm to direct stimulation was not affected by triethyl tin ($1 \times 10^{-5}$ M) or trimethyl tin ($5 \times 10^{-5}$ M) until there had been a gross depression of the responses to indirect stimulation. Diethyl tin dichloride ($1.5 \times 10^{-4}$ M) had no effect on the tetanic response to phrenic nerve stimulation, but after 5 min. exposure it produced an irreversible contracture.

Trimethyl and triethyl tin enhanced the action of both (+)-tubocurarine and decamethonium on the diaphragm. In a concentration of $1 \times 10^{-7}$ M, triethyl tin had no direct action on the preparation, but within 30 min. it led to a complete failure from a dose of tubocurarine or decamethonium that had previously caused only a 50% reduction in the size of the contraction. The effect in neither case was reversed when the tin was removed. Trimethyl tin had a similar effect in a concentration of $5 \times 10^{-6}$ M, but here the effect was rapidly reversed when the tin was removed. When doses of tubocurarine were added at 10 min. intervals to a medium always containing the tin compounds, the enhancing effect of the tin was immediately detectable, but became progressively greater and did not reach a maximum for at least 30 min. with the lowest effective concentrations used. Higher concentrations of tin caused more rapid changes in the response to tubocurarine or decamethonium.

*Frog Rectus Muscle.*—On the frog rectus muscle triethyl tin caused a contracture in concentrations above $5 \times 10^{-6}$ M. Trimethyl tin had a similar action above $5 \times 10^{-4}$ M. Their action was irreversible and not antagonized by pentamethonium. In doses below those causing direct effects triethyl and trimethyl tin had no effect on the response of the frog rectus muscle to tubocurarine, acetylcholine or decamethonium.

### *Metabolic Effects of Alkyl Tin Compounds*

The methods available for Sn determination were not sufficiently sensitive to enable the distribution of the relatively small toxic doses of tin to be accurately plotted within the tissues. The brain, lungs, liver, kidney, spleen, muscle, heart, pancreas, fat and blood were examined in a number of rats and rabbits after both the oral and intravenous administration of triethyl tin. The tin appeared to be distributed fairly uniformly throughout the tissues. In the rat the concentration in the blood (20–30 μg./ml.) was maintained during the 3-day period between the injection of the tin and the death of the animal. Over this period no concentration of the tin within any single organ or tissue was detected.

When the higher alkyl and phenyl derivatives were given to rats by mouth in oil, a considerable proportion was excreted in the faeces, and this may partly account for their lower toxicity.

TABLE IV

THE EFFECT OF TRIETHYL TIN SULPHATE (10 MG./KG. I.P.; *p*H 7.4; 37° C.) ON THE BLOOD-SUGAR CONCENTRATION IN NORMAL RATS, AND IN RATS 67 DAYS AFTER ADRENAL MEDULLECTOMY

Each value is the mean from 2–5 animals. The rats were starved overnight before the expt., the injection was given at 10 a.m., after which they had access to food. Control animals were given an equivalent volume of 0.9% NaCl

| Time after Injection (hr.) | Blood Sugar Concn. (mg./100 ml. Blood) | | |
|---|---|---|---|
| | Normal Controls | Norm | Medullectomized |
| 0 | 74 | — | 66 |
| 0·5 | 67 | 121 | 51 |
| 1·0 | 55 | 101 | — |
| 2·0 | 56 | 109 | — |
| 4·0 | 61 | 117 | 73 |
| 6·0 | 61 | 119 | — |
| 24 | 115 | 123 | — |
| 48 | 129 | 105 | — |

A moderate hyperglycaemia followed the intraperitoneal injection of triethyl tin sulphate, but not after adrenal medullectomy (Table IV). The non-protein nitrogen level of the blood was increased to between 68 and 92 mg./100 ml. blood 24 hr. after the intraperitoneal injection of 10 mg./kg. triethyl tin sulphate, and continued at that level until the rat died.

*In vitro*, triethyl tin ($1.4 \times 10^{-3}$ M) caused a 25% decrease in the pseudo-cholinesterase activity of horse serum. In the rat the true and pseudo-cholinesterase activities of the brain and spinal cord were unchanged 4 hr. after the intraperitoneal injection of 10 mg./kg. triethyl tin sulphate. Observation on rats fed triethyl tin hydroxide (20–80 parts per million) showed that severe signs of poisoning could be produced without significant alteration in the true or pseudo-cholinesterase activity of the spinal cord.

The affected hind limb muscles were also examined. It was found that the total acid soluble phosphate, inorganic phosphate, phosphocreatine and nucleotide (7 min. acid hydrolysable) phosphate fractions were unaltered 30 min. and 4 hr. after the injection of 20 mg./kg. and 52 hr. after the injection of 10 mg./kg. triethyl tin sulphate intraperitoneally in rats.

### *Morbid Anatomy of Alkyl Tin Poisoning*

Autopsies on animals given alkyl tin compounds revealed little to the naked eye. After intraperitoneal injection there was a slight increase in the fluid of the peritoneal cavity with occasional small pleural effusions. These latter were more constant and larger after the sodium tin tartrates. A further change after these compounds was in the appearance of the spleen. Its size was within the normal range, but it was pale, with a bluish white tinge ; in one case it was almost white. The concentration of Sn in the tissues of one rabbit which had received a total of 1.16 g./kg. sodium stannitartrate was estimated, and, although only about half the injected tin could be accounted for in the tissues, the concentration in the spleen was about 10 times greater than that in any of the other tissues. These changes in the spleen suggest that, although neutral solutions of the sodium tin tartrates can be prepared, alterations occur soon after injection whereby the double salts are removed from the blood stream by phagocytosis.

Routine histological examination of the main organs in animals given triethyl tin failed to reveal any abnormality. A more detailed study of the central nervous system in these animals is in progress. In the animals fed triethyl tin in the chronic toxicity experiments extensive damage was found in the central nervous system, and an account of this will be given elsewhere (Magee, Stoner, and Barnes, to be published).

## Discussion

Previous work on the alkyl tin compounds, except for that of McCombie and Saunders (1947), has been confined to the triethyl and tetramethyl derivatives. McCombie and Saunders (1947) examined a range of these compounds, but were solely concerned with their sternutatory action. This is present, but not marked, in the members of the series studied here. The only toxicological studies are those of White (1881) and Ungar and Bödlander (1886), who examined the effect of triethyl tin acetate in frogs, rabbits, dogs and cats. Similar results with tetramethyl tin were reported in a note by Seifter (1939a). The present results confirm much of this earlier work. In addition to giving information of their activity in additional species—the rat, guinea-pig, and fowl—it is now possible to consider the comparative toxicity of a series of alkyl tin compounds.

The most active compounds were the tri- and di-alkyl tin compounds. Tetraethyl tin was less active than triethyl tin, but the similarity of its effects after a latent period suggests the possible *in vivo* conversion of the tetra- to the tri-alkyl derivative. The most active members of both the tri- and di-substituted series were the ethyl compounds ; but this is their only similarity, for not only are their toxicological effects different but the antagonistic action of dimercaprol on diethyl tin suggests that it acts through combination with -SH groups. The establishment of diethyl tin as a -SH inhibitor must await further study, as must the position of the mono-alkyl derivatives.

Triethyl tin was the most active of the tri-alkyl compounds, and the central nervous system was the site of the main action. The typical response seems to be that in the rabbit where, after the intravenous injection of triethyl tin, the phases of initial prostration, partial recovery, and final encephalopathy are clearly seen. By suitable modification of dose, route, and member of the series used, it can be reproduced in the rat and fowl. White (1881) and Ungar and Bödlander (1886) had previously shown its occurrence in the dog and cat. Although much of the earlier work was confirmed, diarrhoea was less often found except as a terminal event after the oral administration of the poorly absorbed compounds. The toxicity of the double salt of tin with sodium tartrate was finally confirmed, although the doses required were larger than those used by White (1881) and Ungar and Bödlander (1886). It is difficult to determine from their papers which of the three possible salts they used. In acute experiments the most active compound, sodium stannous tartrate, was apparently twenty times less toxic than triethyl tin, on the basis of Sn content. This may, however, be a serious overestimate of the difference, since these

double salts may, after injection, form particles which are removed from the circulation by the spleen, so reducing the effective concentration. More important than the difference in toxicity level is the fact that these double salts reproduced the delayed effects of trialkyl tin poisoning. The immediate "narcotic" action was peculiar to trialkyl tin.

The mode of action of the trialkyl tin compounds is not known nor, at the moment, can we advance a single hypothesis satisfactory for all the compounds examined. The earlier workers had concluded that the main effect was on the central nervous system, which is certainly the most obvious site of action. Although the tin is not concentrated in the brain or spinal cord the various peripheral effects seem insufficient to account for the general effect. Work on the action of alkyl tin compounds on the metabolism of tissues *in vitro* is in progress, and although no unifying biochemical lesion can yet be postulated it would seem reasonable to suppose that such a lesion has occurred. The absence of gross pathological lesions in any important organ, despite an interval of several days between the administration of a lethal dose of tin and the death of the animal, supports such a concept; nor is the argument disturbed by the demonstration of widespread damage in the central nervous system of *chronically* poisoned rats, for the diffuse nature of the damage suggests that it is the result of a biochemical lesion. The signs of poisoning suggest that whatever part of the metabolic cycle is disturbed by tin this is more vital to the proper functioning of the brain than to that of other organs.

The peripheral actions themselves give little help in the isolation of the lesion. In some respects these actions resemble those of onium ions, to which chemical group these compounds might be considered to belong. However, the dissociation constant of triethyl tin hydroxide is $3.35 \times 10^{-8}$, indicating that only very weak onium ion activity could be expected (Ing, 1936). The action of these compounds on isolated muscle preparations alone and in the presence of (+)-tubocurarine and decamethonium creates further difficulties in their classification into any well-defined pharmacological group. Since they are not without action on the muscle itself it is not even possible to say that their action is limited to the myoneural junction, although they may act there preferentially.

The decreasing toxicity on either side of the triethyl compound is partly a matter of absorption. The striking difference between the compounds when given by mouth is not seen in the guinea-pig, where absorption from the gut appears more efficient, nor is it seen when they are given parenterally.

The reproduction of the late effects of the trialkyl tin compounds by large doses of sodium tin tartrates is the nearest that has been reached to a comparison with inorganic tin compounds. The insolubility of the latter, and of most other tin compounds, in biological fluids is the main reason for their pharmacological inertness. The pharmacological relationship between the trialkyl tin compounds and other forms of tin appears similar to that between such metals as lead, antimony, bismuth and mercury and their trialkyl derivatives (Harnack, 1878; Seifter, 1939b; Sollman and Seifter, 1939; Hunter, Bomford, and Russell, 1940). In all of these the presence of the alkyl groups enhances the toxicity of the metals —apparently by aiding the absorption of the metal and its distribution to the site of action. Whether the whole difference between the toxicity of the metal and its alkyl derivatives and the immediate effects of the latter can be explained thus is not known. From a practical point of view the striking central nervous system actions of the alkyl tin compounds require that they should be handled with care, particularly during manufacture.

## Summary

1. The biological effects of a series of tetra-, tri-, di- and mono- alkyl tin compounds, and of some other organic tin compounds, have been studied in rats, rabbits, guinea-pigs and fowls, in both acute and chronic experiments.

2. The di- and tri- ethyl tin compounds behave differently and only the toxic effects of the former are antagonized by dimercaprol.

3. In acute experiments on rabbits, triethyl tin, the most active compound, produces muscular weakness followed by some recovery, followed in turn by muscular tremors proceeding to convulsions and death. The other species and compounds show variations of this pattern.

4. The outstanding sign of chronic poisoning is muscular weakness.

5. Although there is no evidence that the tin is concentrated in any particular organ the main site of action of these alkyl compounds appears to be in the central nervous system.

6. Since the delayed effects of triethyl tin poisoning are reproduced by large doses of sodium tin tartrate it is thought that the pharmacological relationship between the triethyl tin compounds and other forms of tin is similar to that between other metals (Pb, Hg, Sb) and their alkyl derivatives.

7. Because of their marked effect on the central nervous system caution is advised in the handling of these compounds.

We wish to thank Mr. A. N. Davison for the cholinesterase determinations, Dr. W. N. Aldridge for the measurement of the dissociation constant of triethyl tin hydroxide, and Dr. P. N. Magee for help with the histology. Our thanks are also due to Miss P. Harrison and Messrs. J. A. E. Jarvis and K. Lorenzen for their technical assistance.

Mr. W. R. Lewis and Dr. J. W. Price, of the Tin Research Institute, supplied us with most of the compounds used in this work, and we are grateful to them for their co-operation.

## Addendum

Since this paper was submitted for publication it has come to our notice that there have recently been 110 deaths in France in cases of furunculosis treated orally with a proprietary preparation containing diethyl tin diiodide dissolved in linoleic acid. The total amount of diethyl tin diiodide consumed by these patients appears to have been about 3.0 g.

Our thanks are due to M. Vaille, of the French Ministry of Health, and Dr. Le Breton, of the Institut Medico-Legal, Paris, for information about patients receiving diethyl tin.

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# A CORRELATION BETWEEN CHOLINESTERASE INHIBITION AND INCREASE IN MUSCLE TONE IN RABBIT DUODENUM

BY

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Experiments with anticholinesterases on whole animals have suggested that symptoms of acetylcholine (ACh) accumulation do not appear until 50–80% of the true (aceto-) cholinesterase activity of the tissues has been inhibited, total inhibition of pseudo- (butyro-) cholinesterase being without effect (Gunter and Mendel, 1945; Hawkins and Gunter, 1946; Koelle and Gilman, 1946; Mazur and Bodanzky, 1946; Hawkins and Mendel, 1947; Nachmansohn and Feld, 1947; Riker and Wescoe, 1949). In contrast, experiments with isolated organs (Koelle, Koelle, and Friedenwald, 1950; Burn and Kottegoda, 1953) suggested that anticholinesterases modify function in much lower concentrations than are necessary to produce 50% inhibition of true cholinesterase and that inhibition of pseudo-cholinesterase alone can produce signs of accumulation of ACh.

The work to be described in this paper was designed to investigate the action of anticholinesterases on the isolated duodenum of the rabbit. The responses of preparations of intact duodenum and of the longitudinal muscle coat (prepared according to Ambache, 1954) to different concentrations of eserine and dyflos (di*iso*propyl phosphorofluoridate, DFP) have been recorded. The distribution of true and pseudo-cholinesterase in the intestinal wall has been investigated manometrically using different substrates, and the inhibition by eserine and DFP of cholinesterase activity in longitudinal muscle homogenates has been determined. In addition, estimations have been made of the residual cholinesterase activity in muscle strips exposed to the action of DFP before homogenizing the tissue.

## METHODS

Rabbits were killed by a blow on the neck followed by bleeding from the severed carotid arteries.

*Isolated Intestine Preparations*

Both whole intestine preparations and longitudinal muscle preparations were suspended in Krebs's bicarbonate solution oxygenated with $O_2$ containing 5% $CO_2$ at 34° C. The solution was made up as follows: NaCl 6.6 g., KCl 0.35 g., $CaCl_2$ 0.28 g. (1.41 ml. 20% soln.), $KH_2PO_4$ 0.162 g. (1.62 ml. 10% soln.), $MgSO_4,7H_2O$ 0.294 g. (2.94 ml. 10% soln.), dextrose 2.08 g., $NaHCO_3$ 2.1 g., distilled water 1 litre. This was used in preference to Locke's or Tyrode's solution so as to avoid effects which may be due to loss of cholinesterase activity resulting from changes in *p*H (Bülbring, Kottegoda and Shelley, 1954). (In that paper errors occurred in the formula given for the modified Krebs's solution used; it contained $NaH_2PO_4$ 0.825 g. in 5 l. and the $MgCl_2$ soln. was 1%.)

*Intact Duodenum Preparations.*—The duodenum was severed near the pyloric sphincter and about 9 in. was removed, washed thoroughly in cold Krebs's solution, and dissected free from fat and mesentery. This piece was then divided into 4 cm. lengths and each was numbered to indicate its original position with respect to the stomach. Each piece was suspended in turn with the lumen closed at both ends in a 30 ml. bath. Its movements were recorded on a smoked drum. Each piece was treated with a single dose of anticholinesterase for 30 min., and the response to a given dose of ACh before and after administration of the anticholinesterase was recorded. In comparing the responses to different doses of anticholinesterase, care was taken to ensure that the initial tension was the same for each successive piece of duodenum. Those pieces not in use were kept in Krebs's solution at room temperature, and the order in which they were used was changed in each experiment. On transferring them to Krebs's solution at 34° C., preparations up to 6 hr. old behaved normally, though the pendular movement was not always as vigorous as that of fresh preparations.

*Longitudinal Muscle Strips.*—These were prepared according to Ambache (1954). When free from the rest of the duodenum, the longitudinal muscle immediately contracted to about a third of its original length. 2.5 cm. lengths of this material were suspended in a 30 ml. bath and their movements were recorded.

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The preparations took some time to relax, and pendular movement seldom appeared before 1 hr. after setting up. Several different concentrations of anticholinesterase were tested on each preparation and the responses to ACh were recorded as before. Active preparations were never obtained unless the duodenum had been allowed to cool to room temperature or slightly below before stripping off the muscle. The longitudinal muscle coat in the rabbit is only 2% by weight of the total intestinal wall, and it appears to be more resistant to the effects of stretching at low temperatures. A similar observation was made by Gunn and Underhill (1914) with circular muscle preparations from the cat intestine.

*Anticholinesterases*

A stock solution of DFP in propylene glycol and a freshly prepared solution of eserine sulphate in water were diluted just before use with 0.9% saline for isolated organ experiments or with distilled water for enzyme experiments.

*Enzyme Preparations*

Tissue homogenates were prepared from frozen tissue which was first chopped finely with the aid of a McIlwain chopper (McIlwain and Buddle, 1953) and then homogenized with dextrose-free Krebs's solution in a Potter-Elvehjem all-glass homogenizer. The homogenates, final concentration 100 mg./ml., were stored at −15° C. Both duodenum and ileum were used. Homogenates of intact intestine, intestine which had had the villi and muscularis mucosae removed by scraping the inner surface with a blunt scalpel, circular muscle coat, and longitudinal muscle coat, were prepared. The longitudinal and circular muscle coats could be separated from the rest of the intestinal wall more readily in tissue which had been frozen than in fresh tissue. Lengths of previously frozen intestine were pinned out flat in Krebs's solution with the inner surface exposed. On scraping this surface vigorously with a scalpel, the longitudinal muscle coat on the outer surface became partially detached and could easily be stripped away. Circular muscle could be obtained by stripping away the other layers, but it tended to stick to the submucosa. The nature of the different layers was confirmed by histological examination.

*Manometric Technique*

Warburg manometers with micro-flasks containing a total fluid volume of 0.8 ml. were used. The tissue was incubated in nitrogen containing 5% $CO_2$ at 37.5° C. Readings were taken at 5-min. intervals for at least 30 min. after the addition of substrate to enzyme, and cholinesterase activity was calculated from the initial slope of the curve of carbon dioxide production. Acetylcholine bromide (ACh), methacholine chloride (MCh), benzoylcholine chloride (BzCh), and butyrylcholine chloride (BuCh) were used as substrates in final concentrations of 0.025 M, 0.0375 M, 0.0075 M, and 0.025 M respectively. Inhibitors were incubated with the enzyme for 30 min. at room temperature plus 15 min. at 37.5° C. before adding the substrate.

*Estimation of Residual Cholinesterase Activity in Muscle Strips Treated with DFP before Homogenizing*

After 30 min. in the presence of DFP the tissue was washed in ice-cold Krebs's solution, dried on filter paper, weighed, frozen, and then chopped rapidly. The mince was homogenized in the maximum possible volume of ice-cold saline to give a homogenate containing 100 mg. tissue/ml. The whole procedure took not more than 15 min. and was repeated on a similar muscle strip which had not been treated with DFP. The cholinesterase activities of the two homogenates were compared using MCh and BuCh as substrates so as to obtain an estimate of the inhibition of true and pseudo-cholinesterase in the intact tissue.

## RESULTS

### *The Action of Eserine and Dyflos on the Isolated Intact Duodenum of the Rabbit and on Preparations of the Longitudinal Muscle Coat*

(*a*) *Intact Duodenum.*—The results obtained were qualitatively similar to those described by earlier workers. A shortening or "increase in tone" of the muscle occurred in response to eserine sulphate $2.7 \times 10^{-8}$ M to $2.7 \times 10^{-4}$ M and to DFP $10^{-8}$ to $10^{-5}$ M. The effect increased with eserine concentration from $2.7 \times 10^{-8}$ M to $2.7 \times 10^{-6}$ M; but in two experiments the effect was smaller in the presence of eserine $2.7 \times 10^{-5}$ M; in a third experiment a smaller effect was only obtained with eserine $2.7 \times 10^{-4}$ M. Similar results were obtained with DFP, a peak effect being observed at $10^{-5}$ M.

With low concentrations of anticholinesterases the increase in tone was achieved gradually and was sometimes accompanied by an increase in the amplitude of the pendular movements. As the anticholinesterase concentration increased, the increase in tone reached its maximum more rapidly and the pendular movement diminished. No effect was observed on the rate of the pendular movement until the peak concentrations were reached and the pendular movement ceased altogether. No difference in sensitivity to anticholinesterases was observed in the different regions of duodenum tested.

(*b*) *Longitudinal Muscle Strips.*—The results obtained with preparations of the longitudinal muscle coat were similar to those described for the intact duodenum. Pendular movement was irregular and not so vigorous as in the intact duodenum, but, once established, it could be maintained for long periods (at least 8 hr.).

Fig. 1 illustrates the responses to ACh and eserine of a typical preparation. This gave graded

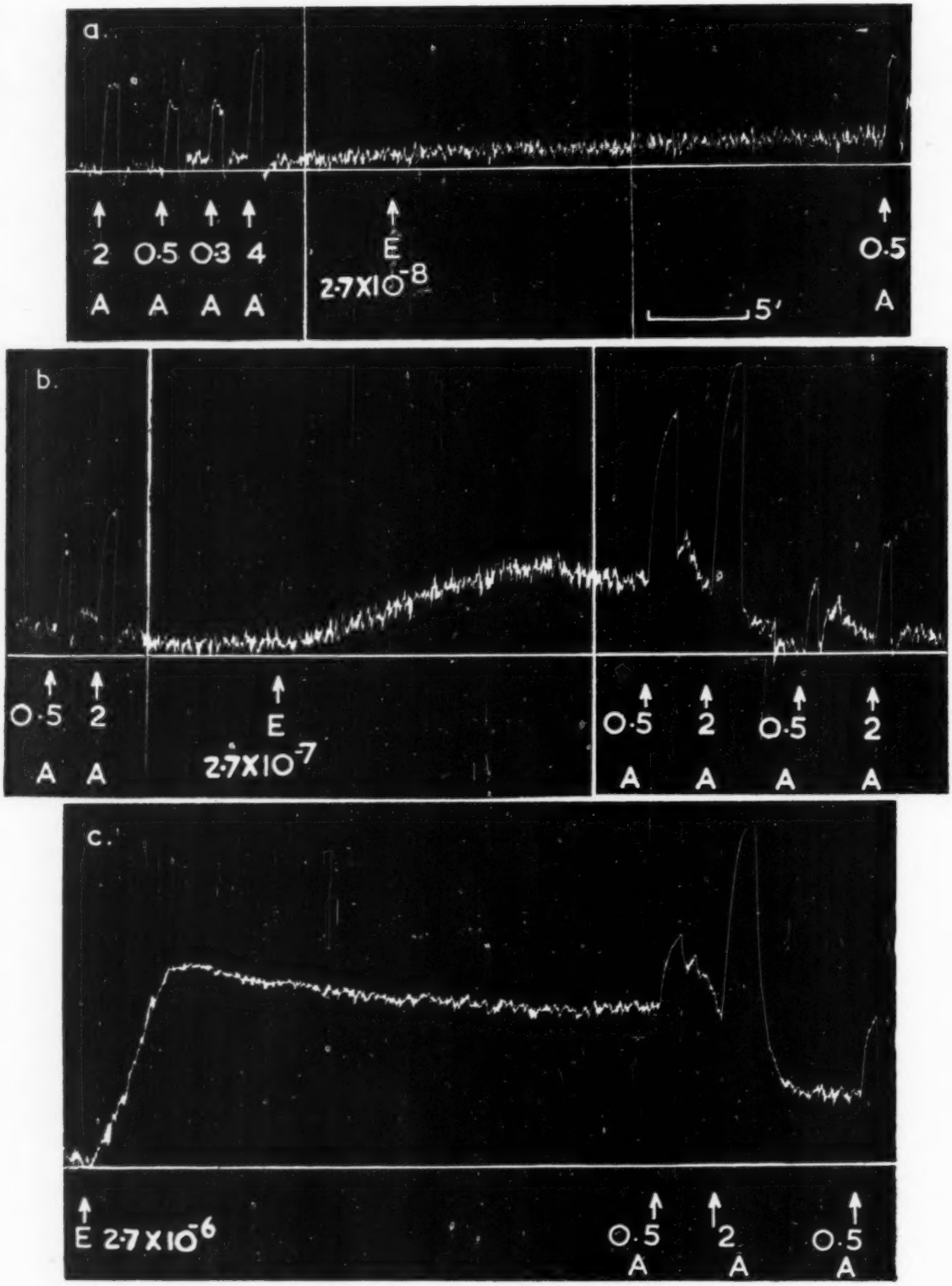


FIG. 1.—Rabbit duodenum longitudinal muscle layer: the effect of increasing molar concn. of eserine (E) on muscle tone and the response to acetylcholine (A, doses in μg.).

contractions in response to doses of ACh varying from 0.3 $\mu$g. to 4.0 $\mu$g. Eserine $2.7 \times 10^{-8}$ M, $2.7 \times 10^{-7}$ M, and $2.7 \times 10^{-6}$ M (Fig. 1, a, b, and c) produced progressively greater increases in tone and potentiation of the response to ACh ; both the tone and the ACh response returned to normal after washing out the eserine. In two other experiments eserine $2.7 \times 10^{-8}$ M had no effect, but similar responses were obtained with eserine $2.7 \times 10^{-7}$ M and $2.7 \times 10^{-6}$ M. As in experiments with intact duodenum preparations, eserine $2.7 \times 10^{-5}$ M and $2.7 \times 10^{-4}$ M produced smaller increases in tone, but in the two experiments in which eserine $2.7 \times 10^{-4}$ M was tested the initial increase in tone was followed by relaxation of the muscle. In the presence of the higher concentrations of eserine the response to ACh disappeared, and in one experiment, the only one in which ACh was administered in the presence of eserine $2.7 \times 10^{-4}$ M, 0.5 $\mu$g. ACh caused relaxation of the longitudinal muscle preparation. No such effect was ever observed with intact duodenum preparations. The close similarity between the response to eserine of the two types of preparation is shown in Fig. 2, where the changes in tone after various doses of eserine are represented graphically.

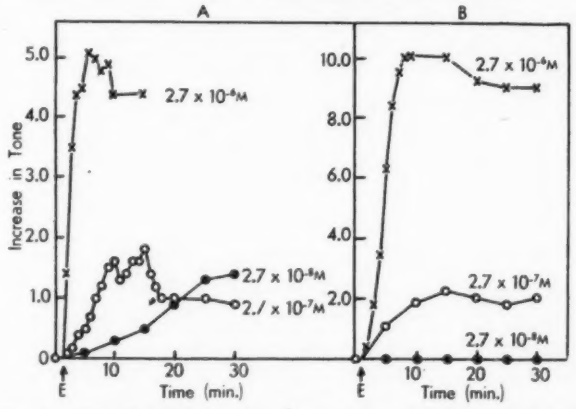


FIG. 2.—The effect of increasing concn. of eserine (E) on muscle tone in preparations of intact rabbit duodenum (A) and of a single longitudinal muscle strip (B). Ordinates: increase in tone, measured as change in level of record in cm. Abscissae: time in min.

Fig. 3 illustrates the effects of DFP in a typical experiment. Concentrations of $10^{-7}$ M, $10^{-6}$ M, and $10^{-5}$ M produced progressively greater increases in tone, and potentiated the effects of ACh. Similar results were obtained in five other experiments. Unlike the effects of eserine, those produced by DFP persisted after washing. The potentiation of the response to ACh remained after washing out the DFP, and, although repeated washing sometimes restored the tone to its original level, a further period without washing usually resulted in a slow return to the level reached in the presence of DFP. As in the experiments with intact duodenum, DFP $10^{-4}$ M in four out of five experiments had a smaller effect than DFP $10^{-5}$ M, and therefore produced a decrease in tone. In two experiments DFP $10^{-3}$ M produced a large decrease in tone, to a level only slightly above normal. These effects are also illustrated in Fig. 3c, where DFP $10^{-4}$ M had no effect but DFP $10^{-3}$ M produced a decrease in tone. A reversal of the response to ACh was never observed in the presence of DFP. Fig. 4 shows the similarity between the response of longitudinal muscle and of intact duodenum preparations to various doses of DFP.

An attempt to summarize the effects of eserine and DFP on the tone of intact duodenum and longitudinal muscle preparations has been made in Table I. In every experiment the maximum change in level of the base line in response to

TABLE I

THE EFFECT OF ESERINE AND DFP ON THE TONE OF THE ISOLATED RABBIT DUODENUM AND OF ISOLATED LONGITUDINAL MUSCLE STRIPS

| Eserine (Molar Concn.) | Increase in Tone as Mean % of Maximum | | DFP (Molar Concn.) | Increase in Tone as Mean % of Maximum | |
|---|---|---|---|---|---|
| | Intact Duodenum | Longitudinal Muscle | | Intact Duodenum | Longitudinal Muscle |
| $2{\cdot}7 \times 10^{-8}$ | 27·5 | 3·0 | $10^{-8}$ | — | 0 |
| $2{\cdot}7 \times 10^{-7}$ | 50·0 | 34·0 | $10^{-7}$ | 18·5 | 18·5 |
| $2{\cdot}7 \times 10^{-6}$ | 100·0 | 100 0 | $10^{-6}$ | 87·5 | 71·0 |
| $2{\cdot}7 \times 10^{-5}$ | 93·0 | 78·0 | $10^{-5}$ | 100·0 | 100·0 |
| $2{\cdot}7 \times 10^{-4}$ | 91·0 | 35·0 | $10^{-4}$ | — | 73·0 |
| No. of expts. | 3 | 3 | No. of expts. | 2 | 6 |

each dose of anticholinesterase was expressed as a percentage of the peak value, observed with eserine $2.7 \times 10^{-6}$ M and with DFP $10^{-5}$ M. For instance, in one experiment, concentrations of DFP $10^{-7}$, $10^{-6}$, and $10^{-5}$ M produced changes in level of the base line of 0.6, 3.3, and 4.5 cm.; the percentage changes were therefore expressed as 13.5, 74, and 100. As might be expected, there was some variation in different experiments, but the mean values (Table I) illustrate the similarity between the effects of eserine and DFP. Moreover, the effects of DFP are quantitatively similar on the two preparations.

An attempt to establish a quantitative relationship between the potentiation of the response to ACh and the concentration of anticholinesterase was unsuccessful. Although the degree of potentiation increased with rising anticholinesterase concentration, this was often obscured by the

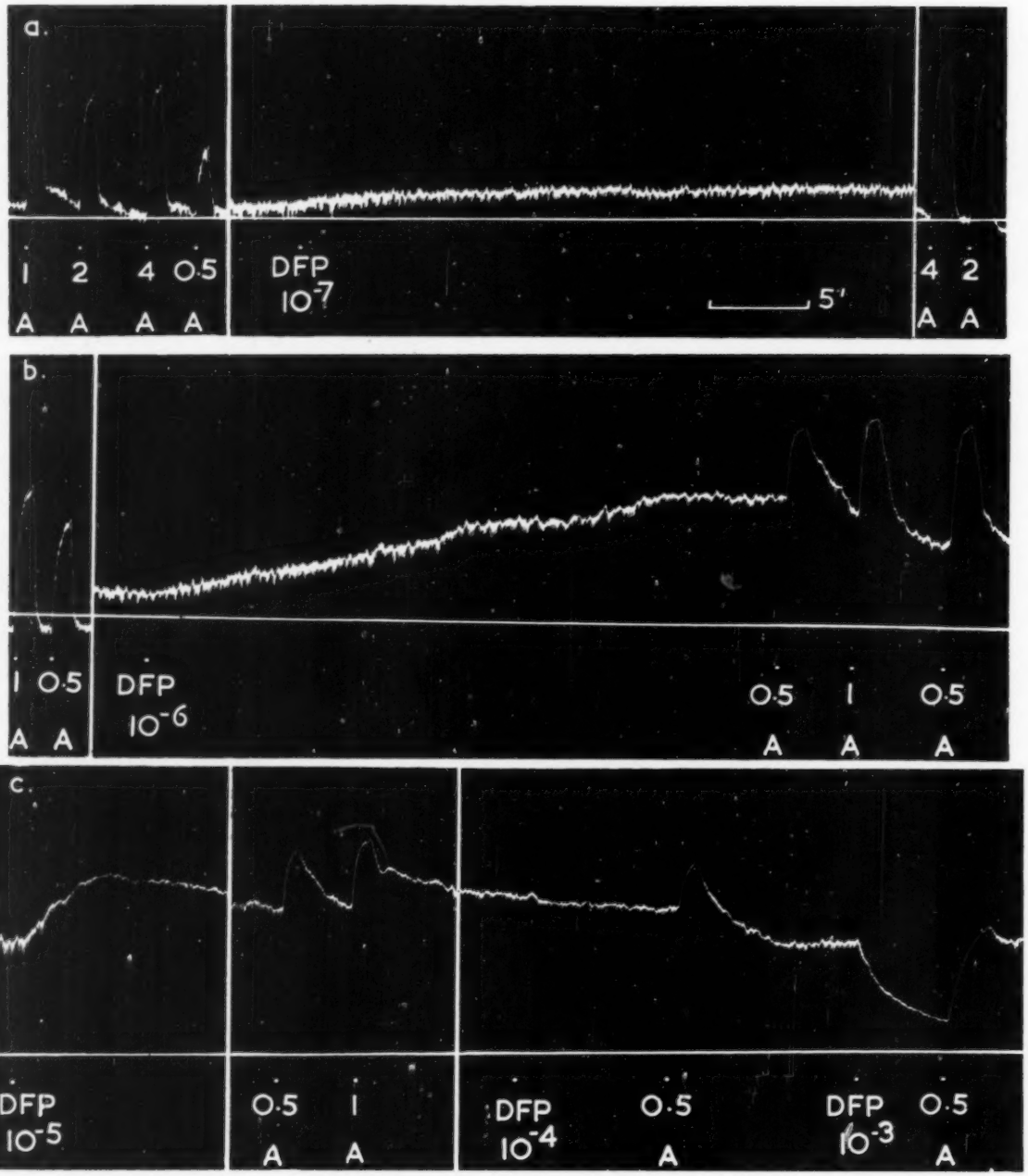


FIG. 3.—Rabbit duodenum longitudinal muscle layer: the effect of increasing molar concn. of DFP on muscle tone and the response to acetylcholine (A, doses in μg.).

change in tone of the preparation, particularly in the presence of high concentrations of anticholinesterase.

*The Distribution of Cholinesterase Activity in the Intestinal Wall*

The true and pseudo-cholinesterase activity of homogenates of intact intestine, as measured by the rates of hydrolysis of MCh and BzCh, were low and the homogenates were so viscous that the results were difficult to duplicate. Homogenates of the circular and longitudinal muscle layers were non-viscous and more active. The rates of hydrolysis of four different choline esters by the various homogenates are summarized in Table II.

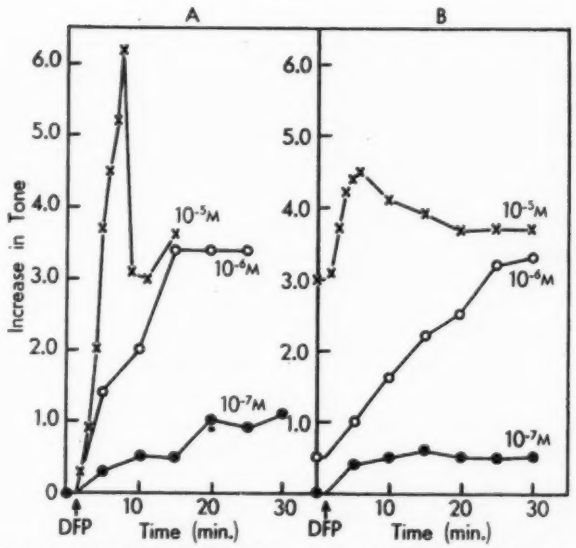


FIG. 4.—The effect of increasing concn. of DFP on muscle tone in preparations of intact rabbit duodenum (A) and of a single longitudinal muscle strip (B). Ordinates: increase in tone, measured as change in level of record in cm. Abscissae: time in min.

The results suggest that true and pseudo-cholinesterase are most highly concentrated in the longitudinal and circular muscle layers of the intestinal wall. Moreover, the longitudinal muscle layer has twice the true cholinesterase activity and nearly three times the pseudo-cholinesterase activity of the circular layer. It is known, and histological examination has confirmed, that most of the cells and fibres of Auerbach's plexus adhere to the longitudinal muscle layer, and this probably accounts for its greater cholinesterase activity.

Homogenates of scraped intestine had a lower pseudo-cholinesterase activity than homogenates of whole intestine, suggesting that the mucosa which had been removed is rich in pseudo-cholinesterase; it is probable that the muscle coats account for the residual pseudo-cholinesterase activity of the scraped intestine. In contrast, true cholinesterase activity was not diminished by removing the mucosa, and the activity of the muscle coats—which are not more than 5% of the weight of the intestinal wall—was insufficient to account for the total activity in whole and scraped intestine. These results suggest that pseudo-cholinesterase activity is concentrated in the muscle coats and in the mucosa, whereas true cholinesterase is present throughout the intestinal wall.

TABLE II

CHOLINESTERASE ACTIVITY IN THE DIFFERENT LAYERS OF THE RABBIT SMALL INTESTINE

| Source of Enzyme | Expt. | Rate of Hydrolysis of Substrate $\mu$l. $CO_2$/g. Tissue/Hr. | | | |
|---|---|---|---|---|---|
| | | ACh 0·025M | MCh 0·0375M | BzCh 0·0075M | BuCh 0·025M |
| Intact small intestine | 1 | 1,040 | 405 | 200 | |
| | 2 | 1,530 | 500 | 250 | |
| | Mean | 1,285 | 453 | 225 | |
| Scraped small intestine, i.e., longitudinal muscle+circular muscle+submucosa | 3 | 1,160 | | | |
| | 4 | 1,400 | | | |
| | 5 | 980 | | | |
| | 6 | 1,280 | | | |
| | 7 | 785 | 560 | 50 | |
| | 8 | 1,330 | 560 | 0 | |
| | 9 | 1,370 | 450 | 110 | |
| | Mean | 1,186 | 523 | 53 | |
| Circular muscle .. | 10 | 2,000 | | | |
| | 11 | 3,200 | 845 | 310 | |
| | Mean | 2,600 | 845 | 310 | |
| Longitudinal muscle | 12 | 5,100 | | | |
| | 13 | 5,680 | 1,720 | 880 | |
| | 14 | 7,720 | 2,260 | 740 | 5,440 |
| | 15 | 6,560 | 2,640 | | 4,920 |
| | 16 | | 1,570 | | 7,720 |
| | 17 | 5,080 | 1,540 | | 5,840 |
| | Mean | 6,028 | 1,946 | 810 | 5,980 |

Expts. 2 and 9 were performed with tissue from the same rabbit. Expts. 10 and 12, 11 and 13 were performed with tissue from the same piece of intestine.

*The Inhibition of True and Pseudo-cholinesterase in the Longitudinal Muscle Layer of Rabbit Intestine by Eserine and Dyflos*

The results with eserine and DFP as inhibitors of the cholinesterase in homogenates of the longitudinal muscle layer are shown in Tables III and IV. The mean results for each substrate have been calculated from three experiments, excepting those for BzCh, which are from one experiment. Table III also contains results obtained with homogenates of scraped intestine.

Table IV includes estimates of the inhibition by DFP of true and pseudo-cholinesterase in the intact longitudinal muscle strip. These were

TABLE III

INHIBITION OF THE CHOLINESTERASES OF RABBIT SMALL INTESTINE HOMOGENATES BY ESERINE

| Eserine Sulphate (Molar Concn.) | % Inhibition of Hydrolysis of Substrates Indicated | | | | |
|---|---|---|---|---|---|
| | Scraped Intestine | Longitudinal Muscle Layer | | | |
| | ACh 0·025M | ACh 0·025M | MCh 0·0375M | BzCh 0·0075M | BuCh 0·025M |
| $2·7 \times 10^{-8}$ | 2·0 | 8·0 | 4·0 | 35·0 | 1·5 |
| $1·35 \times 10^{-7}$ | 12·0 | 8·0 | 10·0 | 34·0 | 12·5 |
| $2·7 \times 10^{-7}$ | 21·0 | 32·0 | 29·5 | 45·0 | 25·5 |
| $1·35 \times 10^{-6}$ | 48·0 | 55·0 | 47·5 | 65·0 | 51·5 |
| $2·7 \times 10^{-6}$ | 58·0 | 69·0 | 70·0 | 88·0 | 67·0 |
| $1·35 \times 10^{-5}$ | 84·5 | 86·0 | 91·5 | 93·0 | 82·0 |
| $2·7 \times 10^{-5}$ | 98·5 | 98·0 | 99·0 | 100·0 | 97·0 |
| $1·35 \times 10^{-4}$ | 100·0 | 100·0 | 100·0 | 100·0 | 99·0 |
| $2·7 \times 10^{-4}$ | 100·0 | 100·0 | 100·0 | 100·0 | 99·0 |
| I 50 | $1·9 \times 10^{-6}$ | $1·35 \times 10^{-6}$ | $1·5 \times 10^{-6}$ | $3·05 \times 10^{-7}$ | $1·6 \times 10^{-6}$ |

I 50 = molar concn. of eserine sulphate necessary for 50% inhibition of substrate hydrolysis.

TABLE IV

INHIBITION OF THE CHOLINESTERASES OF THE LONGITUDINAL MUSCLE OF RABBIT SMALL INTESTINE BY DFP ADDED TO INTACT MUSCLE STRIPS OR TO HOMOGENATES

| DFP (Molar Concn.) | % Inhibition of Hydrolysis of Substrate Indicated | | | | |
|---|---|---|---|---|---|
| | ACh 0·025M | MCh 0·0375M | | BuCh 0·025M | |
| | Homogenate | Homogenate | Intact Muscle Strip | Homogenate | Intact Muscle Strip |
| $10^{-9}$ | 13·0 | | | 0·5 | |
| $5\times10^{-9}$ | 19·0 | | | 7·0 | |
| $10^{-8}$ | 24·0 | 6·0 | 5·0 | 19·5 | 41·0 |
| $5\times10^{-8}$ | 35·0 | 10·5 | | 58·0 | |
| $10^{-7}$ | 54·0 | 19·0 | 10·0 | 88·0 | 74·5 |
| $5\times10^{-7}$ | 73·0 | 39·0 | | 95·0 | |
| $10^{-6}$ | 95·0 | 65·0 | 59·5 | 99·0 | 93·5 |
| $5\times10^{-6}$ | 100·0 | 94·0 | | 100·0 | |
| $10^{-5}$ | 100·0 | 100·0 | 100·0 | 100·0 | 99·5 |
| I 50 | $9\cdot5\times10^{-8}$ | $7\cdot5\times10^{-7}$ | $9\cdot0\times10^{-7}$ | $4\cdot0\times10^{-8}$ | $2\cdot5\times10^{-8}$ |

I 50 = molar concn. of DFP necessary for 50% inhibition of substrate hydrolysis.

obtained by comparing the residual cholinesterase activity after treating the intact strip with DFP for 30 min. with the activity of untreated control strips. Each estimate is the mean of two experiments, except that for DFP $10^{-5}$ M, which is the result of a single experiment. In most experiments the estimated inhibition of true and pseudo-cholinesterase obtained by treating the intact longitudinal muscle strip with DFP was similar to that obtained by adding DFP to the muscle homogenate.

### *Correlation of Increase in Tone in Longitudinal Muscle with Cholinesterase Inhibition*

Figs. 5 and 6 represent an attempt to correlate the percentage inhibition of true and pseudo-cholinesterase with the corresponding increase in tone of longitudinal muscle preparations calculated

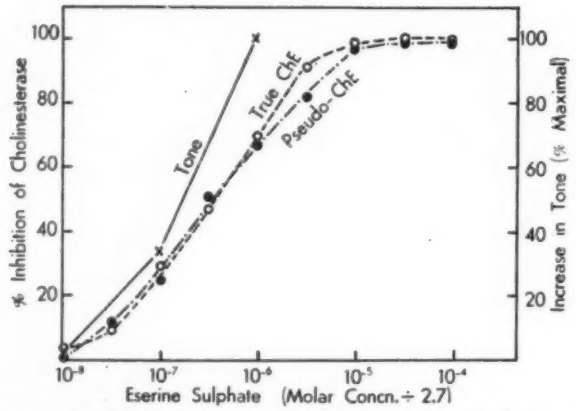


FIG. 5.—The effect of increasing concn. of eserine on cholinesterase (ChE) activity and tone in the longitudinal muscle of the rabbit small intestine.

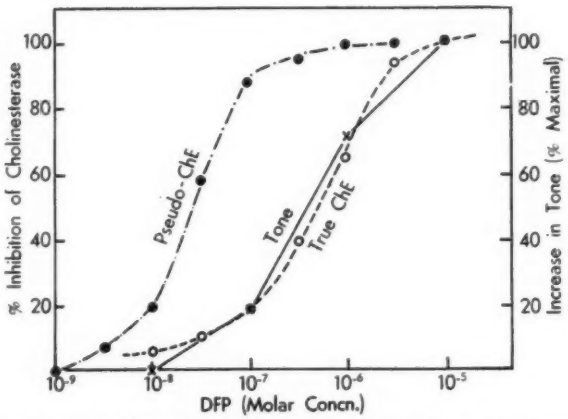


FIG. 6.—The effect of increasing concn. of DFP on cholinesterase (ChE) activity and tone in the longitudinal muscle of the rabbit small intestine.

as in Table I. In Fig. 5 the percentage increase in tone produced by a given concentration of eserine is always greater than the percentage inhibition of either true or pseudo-cholinesterase, but the curves are almost parallel, suggesting a relationship between increase in tone and inhibition of cholinesterase activity. As can be seen in Fig. 6, this correlation is more dramatic when DFP is the inhibitor. Since pseudo-cholinesterase is more readily inhibited than true cholinesterase by DFP, the inhibition curves for the two enzymes are quite distinct and the curve of increase in tone is exactly superimposed upon the inhibition curve for true cholinesterase.

## DISCUSSION

The effects of eserine and DFP on the tone of isolated intestine preparations from several species have been investigated by a number of workers (see Heathcote, 1932; Feldberg and Solandt, 1942; Bacq, 1947; Feldberg and Lin, 1949; Koelle, Koelle, and Friedenwald, 1950), but little attempt has been made to relate the intensity of the effect to the concentration of anticholinesterase. Bacq found that isolated cat or rabbit intestine would not respond to a second dose of eserine or DFP, and Koelle *et al.* were unable to obtain a quantitative relationship between DFP concentration and the response of isolated cat intestine.

In the present work the effects of previous doses of anticholinesterase have been avoided, by testing each dose of eserine or DFP on a different piece of rabbit duodenum, and it was observed that the magnitude of the response increased with rising anticholinesterase concentration to a maximum in the presence of eserine $2.7\times10^{-6}$ M or DFP $10^{-5}$ M. When the responses to lower concentrations of

anticholinesterase were expressed as percentages of the maximum response, there was quantitative agreement between different experiments. Similar results were obtained when successive doses of anticholinesterase were tested on the same longitudinal muscle preparation. As had been observed by Heathcote (1932), still higher concentrations of eserine produced smaller increases in tone and this was also true of DFP. These effects are probably due to actions of eserine and DFP distinct from their anticholinesterase action.

There has been no previous investigation of the rates of hydrolysis of choline esters by the different layers of the intestinal wall in the rabbit, but Koelle (1953) gives comparable figures for the hydrolysis of ACh, MCh, and BzCh by homogenates of whole intestine. Koelle investigated the distribution of true and pseudo-cholinesterase in the rabbit intestine by histochemical methods, and his results are similar to those obtained in the present work using manometric methods. He found that true cholinesterase occurred in nervous tissue throughout the intestinal wall, and in low concentrations in the interstitial cells of the muscle layers ; pseudo-cholinesterase was mainly present in the interstitial cells and in the mucosa, but was also found in ganglion cells. Koelle also investigated the inhibition of rabbit cholinesterases by eserine and DFP, and his results are confirmed in this paper (see also Mazur and Bodanzky, 1946).

The aim of the present work was to correlate the increase in tone of the intestine preparations with the percentage inhibition of cholinesterase activity. As in all work of this kind, the chief difficulty was to determine the relationship between the inhibition of cholinesterase activity *in vitro* and the inhibition of ACh hydrolysis in the intact tissue.

This is especially difficult when a reversible inhibitor, such as eserine, is used. The addition of substrate to a cholinesterase preparation in the presence of eserine will partially reverse the eserine inhibition of the enzyme, and the extent of this reversal will depend both on the affinity of the enzyme for the substrate and on the concentration of added substrate. For this reason the measurement of cholinesterase inhibition by eserine *in vitro* in the presence of 0.025 M ACh will not give an accurate measure of the inhibition of ACh hydrolysis in the intact tissue, where the substrate concentration is unknown. In the intact tissue the ACh concentration is probably less than 0.025 M and therefore the inhibition of ACh hydrolysis will probably be greater than that measured *in vitro*. Similarly, the measurement of true or pseudo-cholinesterase inhibition by eserine using specific substrates such as MCh, BzCh, and BuCh will not give an accurate measure of the inhibition of ACh hydrolysis by these enzymes either *in vivo* or *in vitro*. This point may be illustrated by the results obtained for the inhibition by eserine of pseudo-cholinesterase in longitudinal muscle homogenates (Table III). When 0.0075 M-BzCh was used as substrate the apparent inhibition of pseudo-cholinesterase activity by a given concentration of eserine was always greater than the corresponding inhibition of ACh or MCh hydrolysis in agreement with earlier observations by Blaschko, Bülbring and Chou (1949). In contrast, when 0.025 M-BuCh was used, the apparent inhibition of pseudo-cholinesterase activity was similar to that of ACh or MCh hydrolysis (see also Fig. 5).

It is preferable, therefore, to use an irreversible inhibitor, such as DFP, in all work of this kind. Mackworth and Webb (1948) have shown that, in contrast to eserine, inhibition of cholinesterase activity by DFP is not reversed by the addition of high substrate concentrations. Therefore, comparison of the rates of hydrolysis of different substrates in the presence of DFP, added at a fixed time interval before the substrate, does give a true measure of the inhibition of the enzyme under investigation, and it can definitely be said that DFP inhibits pseudo-cholinesterase more readily than true cholinesterase. It may also be assumed that the inhibition produced by DFP in the intact tissue is no greater than that produced by adding DFP to the tissue homogenate, and this has been confirmed in the present work.

Another possible source of error is incomplete penetration of the intact tissue by the anticholinesterase. The longitudinal muscle layer of the rabbit intestine is very thin, however (about 120 $\mu$), and penetration should be relatively easy, especially when preparations of isolated longitudinal muscle are used. It has been assumed that, after 30 min. in the presence of eserine or DFP, penetration of the anticholinesterase into this muscle layer is complete. The increase in tone of the longitudinal muscle usually reached a maximum within 30 min. of administering the anticholinesterase ; there was also good agreement between the inhibition of cholinesterase by DFP added to longitudinal muscle homogenates and the cholinesterase inhibition in strips of longitudinal muscle treated for 30 min. with DFP before homogenizing them. Moreover, Koelle, Koelle, and Friedenwald (1950) examined suitably stained sections of cat intestine which, when intact, had been exposed to the action of DFP, and observed that the inhibition of the longitudinal muscle cholinesterase

corresponded to that determined manometrically with homogenates of whole intestine, though the inhibition in the deeper layers was often less.

Koelle *et al.* also made manometric determinations of the residual cholinesterase activity in these segments of cat intestine. They observed that concentrations of DFP which had produced large increases in tone had almost completely inhibited the pseudo-cholinesterase, whereas the true cholinesterase was hardly affected. Since, in addition, they had observed mainly pseudo-cholinesterase in the longitudinal muscle layer (see also Koelle, 1950; 1951), they concluded that there is a close relationship between pseudo-cholinesterase activity and intestinal motility. This conclusion was supported by Burn, Kordik, and Mole (1952), who, having observed diarrhoea in rats exposed to X-rays, tested the reaction of intestinal loops to ACh and found that they responded to much lower concentrations than usual. They observed that the pseudo-cholinesterase activity was reduced to less than half, while the true cholinesterase activity was unchanged.

The results obtained in the present work on the rabbit intestine suggest that true cholinesterase is at least as important as pseudo-cholinesterase. When the percentage increase in tone and the percentage inhibition of true cholinesterase by DFP were plotted against the concentration of DFP, the two curves were superimposed (Fig. 6). Maximum increase in tone coincided with 100% inhibition of true cholinesterase activity, whereas 100% inhibition of pseudo-cholinesterase occurred at a much lower concentration. In contrast, the percentage increase in tone produced by a given dose of eserine was always greater than the percentage inhibition of either cholinesterase (Fig. 5). However, since the inhibition of cholinesterase activity produced by eserine in the intact tissue was probably greater than that measured *in vitro*, it is possible that the increase in tone produced by both eserine and DFP corresponded with true cholinesterase inhibition in the intact tissue though not necessarily with inhibition as measured *in vitro*.

The results recorded here show that symptoms of ACh accumulation can occur when true cholinesterase activity is only partially inhibited. Previous work has suggested that there is a vast excess of true cholinesterase in the tissues, most of which can be inhibited without effect (Gunter and Mendel, 1945; Mazur and Bodanzky, 1946; Koelle and Gilman, 1946; Hawkins and Mendel, 1947; Nachmansohn and Feld, 1947; Hawkins and Gunter, 1949; Hawkins and Mendel, 1949; Riker and Wescoe, 1949), but in the present work an increase in tone in the rabbit intestine was regularly observed, not only with eserine $2.7 \times 10^{-7}$ M, but also with DFP $10^{-7}$ M, a concentration sufficient to produce only 10–20% inhibition of true cholinesterase activity. Similarly, Burn and Kottegoda (1953) observed changes in the rate and amplitude of beat of isolated rabbit auricles in the presence of eserine $2.7 \times 10^{-8}$ M to $2.7 \times 10^{-7}$ M, sufficient to produce not more than 16% inhibition of ACh hydrolysis by auricle homogenates *in vitro* (Shelley, unpublished experiments); but again, since eserine is a reversible inhibitor, the inhibition of cholinesterase activity in the intact tissue was probably greater than that measured with the homogenates.

The ratio of cholinesterase activity to ACh content is not the same for all tissues, and it is possible that tissues with a low cholinesterase activity and a high rate of ACh production do not contain a large excess of cholinesterase. In the rabbit intestine cholinesterase activity is low compared with many tissues, and the rate of ACh production is high (Feldberg and Solandt, 1942; Feldberg and Lin, 1950). Moreover, the ACh content is high compared with other tissues (Chang and Gaddum, 1933), the longitudinal muscle layer containing 10–16 μg./g. (Welsh and Hyde, 1944), an exceptionally high ACh content for a mammalian tissue. The cholinesterase activity of rabbit auricles is also low (Bülbring, Kottegoda, and Shelley, 1954), and, though the rate of ACh synthesis is not high (Bülbring and Burn, 1949), the auricles contain up to 4.0 μg. ACh/g. (Briscoe and Trendelenburg, unpublished experiments). Under these conditions of low cholinesterase activity and high ACh content, it is conceivable that symptoms of ACh accumulation should appear when the degree of cholinesterase inhibition is comparatively slight, and should increase in intensity—as in the rabbit duodenum—until the cholinesterase activity has been reduced to zero.

## Summary

1. The effect of eserine and dyflos (DFP) on tone in the isolated rabbit duodenum and in isolated strips of longitudinal muscle from the rabbit duodenum has been investigated.

2. An increase in tone first appeared with eserine $2.7 \times 10^{-8}$ M or DFP $10^{-7}$ M. This effect increased, with rising anticholinesterase concentration, to a maximum in the presence of eserine $2.7 \times 10^{-6}$ M or DFP $10^{-5}$ M. Above these concentrations the increase in tone declined with rising anticholinesterase concentration.

3. The distribution of true and pseudo-cholinesterase in the different layers of the intestinal wall was investigated manometrically using different substrates. Both enzymes were present in the longitudinal muscle layer, and the cholinesterase activity of this layer was higher than that of any other layer.

4. The inhibition of the true and pseudo-cholinesterase of the longitudinal muscle layer by eserine and DFP was investigated. Pseudo-cholinesterase was more readily inhibited than true cholinesterase by DFP, but, using 0.025 M-BuCh and 0.0375 M-MCh as substrates, both enzymes appeared to be equally inhibited by eserine.

5. An estimate of the inhibition of true and pseudo-cholinesterase activity by DFP in the intact longitudinal muscle strip was obtained by measuring the residual cholinesterase activity in longitudinal muscle strips which had been treated with DFP before homogenizing the tissue. These results were similar to those obtained by treating muscle homogenates with DFP.

6. The results suggest that increase in tone in the longitudinal muscle coat of the rabbit intestine first appears when less than 20% of the true cholinesterase is inhibited and reaches a maximum with total inhibition of true cholinesterase activity.

I should like to thank Miss Sheila Briscoe, of this Department, for estimating the residual cholinesterase activity in muscle strips treated with DFP before homogenizing the tissue, and Dr. E. H. Leach, of the Department of Physiology, University of Oxford, for examining histologically the intestinal preparations. My thanks are also due to Professor J. H. Burn and Dr. Edith Bülbring for much helpful advice and encouragement throughout this work.

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# THE ISOLATED HEART OF *MYA ARENARIA* AS A SENSITIVE PREPARATION FOR THE ASSAY OF ACETYLCHOLINE

BY

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The estimation of acetylcholine (ACh) in biological material requires a method of high sensitivity when the concentration likely to be found is very small. Chemical methods so far reported have little value in this respect, for the minimal amount detectable, 0.04 $\mu$.mole in final solution by Hestrin's method (1949), is relatively large. The sensitivity of biological preparations is considerably higher, and the following threshold values have been reported: frog rectus, 2.0 $\mu$g./100 ml.; frog heart, 1.0 $\mu$g./100 ml.; eserinized dorsal leech muscle, 0.2 $\mu$g./100 ml. (Chang and Gaddum, 1933; Gaddum, 1936). In recent years a new method, using the isolated heart of *Venus mercenaria*, has been described. The sensitivity of this preparation to ACh was recorded by Prosser and Prosser (1937), and the application of the method to ACh assay by Wait (1943). Subsequently, many papers have appeared describing the use of this preparation—for example, those of Welsh (1942), Welsh and Taub (1948), Welsh and Slocombe (1952) and Tower and McEachern (1948). The sensitivity and specificity of the *Venus mercenaria* heart are high, and the threshold values for ACh vary from $10^{-1}$ $\mu$g./100 ml. (Smith and Levin, 1938) to $10^{-3}$ $\mu$g./100 ml. (Prosser and Prosser, 1937; Welsh and Taub, 1948).

The method to be described was developed during the course of an investigation into cerebrospinal fluid (c.s.f.) changes in human subjects after head injury. The presence of small concentrations of ACh in the c.s.f. of such cases has been reported by Bornstein (1946), Tower and McEachern (1949) and Ruge (1954). One purpose of this investigation was to test the hypothesis that the beneficial effects of atropine administration in such cases could be linked with the presence of ACh in the c.s.f. (Ward, 1950). The reported concentrations have been small, usually 1–6 $\mu$g./100 ml. Clearly some more sensitive preparation than frog rectus or dorsal leech muscle was necessary; Tower and McEachern had recommended the isolated heart of *Venus mercenaria* and had assayed ACh in c.s.f. with this preparation.

Enquiries of Dr. Russell at Plymouth and Dr. Ford at Millport showed that *Venus mercenaria* was only occasionally obtainable and that it could not be supplied regularly or in quantity. Dr. Ford, however, very kindly supplied specimens of various molluscs for trial, and amongst these *Mya arenaria* was found to be the most suitable for size and sensitivity.

## METHODS

The method of preparation was essentially the same as that described by Wait (1943), Welsh and Taub (1948), and Tower and McEachern (1948) for *Venus mercenaria*. Slight modifications were made to suit the method to *Mya*.

Regular supplies of *Mya* were obtained from Millport, dispatched by rail packed in seaweed. As soon as they arrived they were stored dry at 4° C. In such conditions they remained alive and active for assay purposes for about two weeks. A few survived for longer periods, but preparations from them tended to be sluggish, irregular in beat, and relatively insensitive.

*Mya arenaria* is a bivalve mollusc averaging 10 cm. in length. The heart is situated just below the hinge, towards the siphon end (Fig. 1a). When taken from the cold chamber they were left for a few minutes in sea-water at 15° C. The shell was then cracked on both sides by a blow just below the hinge, and that portion of the shell lifted off. The heart could be seen beating within the pericardial sac, which was opened widely. In the *Venus* preparation ligatures are recommended at the auriculo-ventricular junction. With *Mya*, however, this method proved difficult, and it was found much easier to ligate each end of the median ventricle, taking in the intestine and a small portion of the ventricular wall (Fig. 1b). The heart was then cut loose—by dividing the auricles, intestine, and dorsal vessels—and was suspended in a chamber of sea-water. This consisted of an inner portion, filled from below and with overflow emptying; in testing c.s.f., a capacity of 5 ml. to the overflow was found to be a convenient size. The chamber was filled from a sea-water reservoir at 15° C. and surrounded by a water jacket. The heart was suspended from a hollow hook through which air was bubbled. The recording apparatus consisted of a light heart lever ink-writing on a continuous paper kymograph.

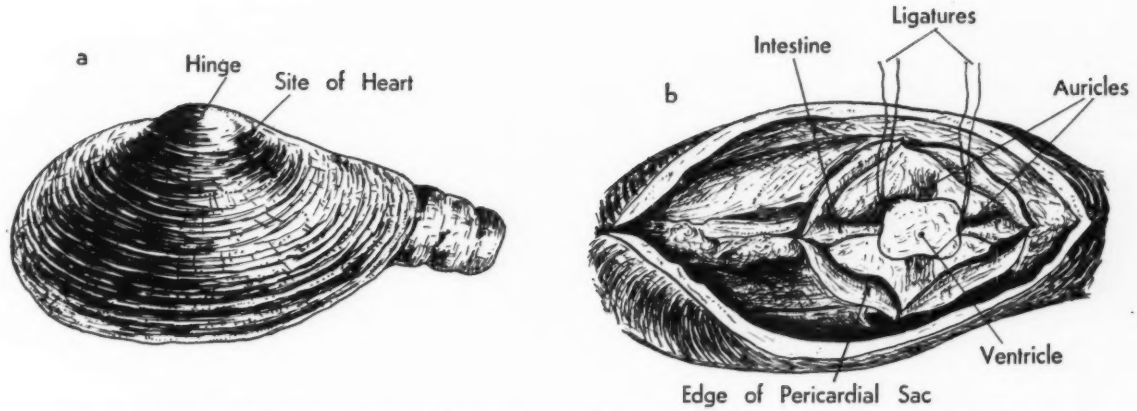


FIG. 1.—Diagrams showing anatomy of the heart region in *Mya arenaria* and the site of application of the ligatures.

A fresh heart started beating within half a minute of setting up ; the beat could be started by tension or by repeated washings with sea-water. In older hearts the beat tended to be slow and irregular at first, but most preparations were beating regularly and ready for use within 15 min. Old hearts that beat irregularly could be revived by the addition of ergometrine maleate. This effect has been described in *Venus* preparations (Welsh and Taub, 1948), and appears to work for *Mya* also. The addition of ergometrine for 2 or 3 min. increased the rate and height of contraction, though sensitivity to ACh was not altered. The ergometrine was then washed out with several changes of sea-water, but the effect persisted for several hours. The preparation could be kept going for 3 to 4 hr. without losing sensitivity : after this period sensitivity to ACh fell off. The test substance was added to the bath with a long hypodermic needle, bent at right angles, on a 1 ml. syringe. After the effect had been recorded the chamber was washed out by overflow with a generous amount of sea-water.

## RESULTS

The isolated heart preparation of *Mya* responded to ACh by a reduced amplitude of ventricular contraction, the effect depending on the dose (see Fig. 2). The full effect was not seen for two or three beats of the heart ; thereafter the heart beat at the new level for several minutes and later tended to recover, though seldom to the previous height. As soon as the ACh was washed out the heart recovered quickly, and often beat at a slightly higher level for some time afterwards. Within two or three minutes, however, the preparation again became stable and further tests could be carried out. With large doses the heart stopped immediately in diastole, and after washing there was some delay in recovery.

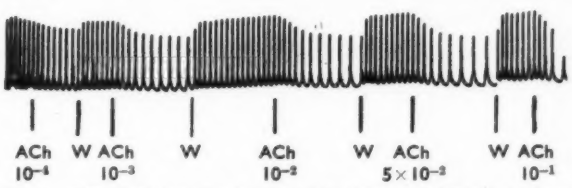


FIG. 2.—Effect of ACh on isolated *Mya* heart. Different doses of ACh (in $\mu$g./100 ml.) produce marked slowing of the heart as well as reduction of amplitude. This was an old preparation. The slowing increases as the dose increases.

The response of *Mya arenaria* differs from that of *Venus mercenaria* in that the reduced ventricular contraction may be accompanied by slowing. This was most marked in old specimens, or in old preparations which were losing their sensitivity ; in such preparations slowing might be the only response. This response, however, is not useless, for the degree of slowing is proportional to dose and may be used as a measure of response together with, or in place of, the effect on ventricular contraction.

The sensitivity of *Mya arenaria* to ACh varied a good deal according to the length of storage and time of year. During most of the year the threshold concentration was $10^{-2}$ to $10^{-3}$ $\mu$g./100 ml. In the months of April and May, however, much greater sensitivity was found in some specimens, and in one batch a response to $10^{-8}$ $\mu$g./100 ml. was obtained.

ACh in very small doses had the effect of increasing slightly the rate and height of ventricular contraction. It was also found that in sluggish hearts after a " stopping " dose of ACh had been administered and washed out the heart beat much better and became more sensitive.

The assay of c.s.f. was done by comparing the effect of doses of ACh with doses of c.s.f. A small chamber was used, for normally only small amounts of c.s.f. were available, the dose used being 0.5 ml. in the 5 ml. chamber. The identity of the test substance was further tested by heating c.s.f. with alkali. After neutralization the previous inhibitory effect was abolished.

*Other Effects.*—The isolated heart of *Mya arenaria* appeared to be highly specific to ACh, as is the heart of *Venus mercenaria*. Up to 2 ml. of c.s.f. could be added to the 5 ml. chamber without effect, and c.s.f. containing protein up to 4 g./100 ml. could be added in 0.5 ml. doses without effect. Human blood in 1 ml. doses had no effect.

Noradrenaline in doses up to 100 $\mu$g./100 ml. had no effect. With larger doses the heart rate increased and tonus increased.

The effects of methacholine, and of benzoylcholine, were essentially similar to that of ACh, but the preparation was considerably less sensitive, the threshold for methacholine being about 10,000 times that for ACh.

Temperature had a marked effect on this preparation. Below 10° C. the heart beat was slow and of low amplitude and the sensitivity to ACh was greatly reduced. Above 20° C. the beat was slow and irregular; again ACh sensitivity was reduced. With *Venus* preparations an optimum temperature of 15° C. was recorded; with *Mya* a temperature slightly above this was found to be more favourable, and the best results were obtained at 17°–18° C.

## Discussion

The isolated heart of *Mya arenaria* is a highly specific preparation for the assay of acetylcholine. It compares favourably in this respect with *Venus mercenaria* and is easily obtainable in this country. It is slightly more sensitive than *Venus* at most times of the year, and in the spring considerably more so. It has the disadvantage that the reduced ventricular contraction is sometimes accompanied by slowing of rate, but as the slowing is proportional to the dose this can be used as a measure of response.

## Summary

1. In the assay of acetylcholine (ACh) in cerebrospinal fluid (c.s.f.) there is need for a highly specific and sensitive preparation. *Venus mercenaria* is such a preparation, but is not obtainable everywhere.

2. The method of preparation and use of the isolated heart of *Mya arenaria*, which is easily obtainable in the British Isles, are described. This heart has a high specificity and sensitivity to ACh, to which it responds by reduction of the amplitude of ventricular contraction with or without slowing. The degree of inhibition or slowing is proportional to the dose of ACh. The threshold concentrations usually lie between $10^{-2}$ and $10^{-3}$ $\mu$g./100 ml.

3. No response is obtained from large doses of normal human c.s.f., highly proteinous c.s.f., or human blood. The effect of ergometrine maleate is to increase the rate and height of ventricular contraction without altering sensitivity to ACh. The effect of noradrenaline is to increase the rate and tonus.

My thanks are due to Dr. E. Ford, of the Scottish Marine Biological Association, for his helpful advice in selecting a suitable preparation, and Dr. F. Neale, of the Queen Elizabeth Hospital, Birmingham, for designing and constructing the heart chambers and hooks.

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*Brit. J. Pharmacol.* (1955), **10**, 39

# THE EFFECTS OF ADRENALINE, NORADRENALINE AND DIHYDROERGOTAMINE ON EXCISED HUMAN MYOMETRIUM

BY

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(RECEIVED JULY 13, 1954)

The response of the uterus to adrenal medullary hormones differs among the various species, and may, in some species, differ in the pregnant and non-pregnant states. In an earlier communication it was shown that adrenaline inhibited, and noradrenaline stimulated, the large co-ordinated contractions of the intact, late pregnant and labouring human uterus (Garrett, 1954). In this paper the responses of excised strips of human myometrium to these hormones are described.

Adrenaline *B.P.* (Epinephrine *U.S.P.*) prepared from natural sources is now known to contain 15 to 20% noradrenaline. Therefore, if previous workers reporting on the action of adrenaline have not stated that they used the pure synthetic substance, it follows that some revision of their work is necessary, lest the presence of noradrenaline as an impurity had vitiated their results.

Greef and Holtz (1951a and b) have repeated the experiments of earlier workers with excised tissue *in vitro*, using pure adrenaline and noradrenaline. They have shown that noradrenaline has a consistent excitatory effect during parturition in the rabbit, dog, guinea-pig and rat. Their observations with adrenaline in these animals simply confirm the findings of past workers, whose results were summarized by Gunn (1944).

A few experiments were done with human tissue in earlier years. Rubsamen and Klingerman (1912) (quoted by Gunn and Scott Russell, 1946) examined strips of myometrium from the pregnant uterus at term, and showed that adrenaline caused a rise in tone and an increase in the individual contractions.

Gunn (1914) examined strips from one non-pregnant uterus. He recorded the spontaneous contractions of the myometrium and found that these were stimulated by adrenaline. These few experiments were repeated on a much larger scale by Gunn and Scott Russell (1946), who showed that adrenaline always stimulated the contractions of excised human myometrium from both pregnant and non-pregnant uteri.

The present work is an attempt to amplify previous findings by examining the response of excised human myometrium to the pure substances (−)-adrenaline and (−)-noradrenaline.

## METHODS

The apparatus for recording the contractions of exsected human myometrium was of the familiar type used for isolated organs, following the method of Gunn and Scott Russell (1946).

The strip of myometrium was suspended in a 10-ml. cylinder containing Ringer-Locke's solution through which a continuous flow of oxygen was bubbled. The composition of the fluid was: NaCl 9 g., KCl 0.42 g., $CaCl_2$ 0.24 g., $NaHCO_3$ 0.2 g., glucose 0.1 g., distilled water to 1,000 ml. The bath was held in a large water jacket at 37° C., and was fed through a narrow coiled glass tube of 25 ml. capacity, so that the specimen could be twice washed without any change in the temperature of the Ringer-Locke's solution. The strip of myometrium in the bath was fixed at one end to a glass rod, and at the other to a light lever recording on a smoked paper. Tracings of these records were made for reproduction here.

In all, 71 strips from 40 uteri were examined. The strips from non-pregnant uteri obtained at hysterectomy were taken vertically from the midline of the anterior surface of the body of the uterus, and were approximately of uniform size, 30 to 40 mm. × 3 mm. × 3 mm. Those from pregnant uteri obtained at caesarean section were cut transversely from the edge of the incision in the lower segment operation and were of variable size.

Most specimens were examined within a few hours of their removal, but sometimes they were stored in Locke's solution in a temperature-controlled refrigerator, at 10° C., for up to 48 hr. before records were obtained.

As Gunn and Scott Russell found, spontaneous rhythm was not established for a variable time after

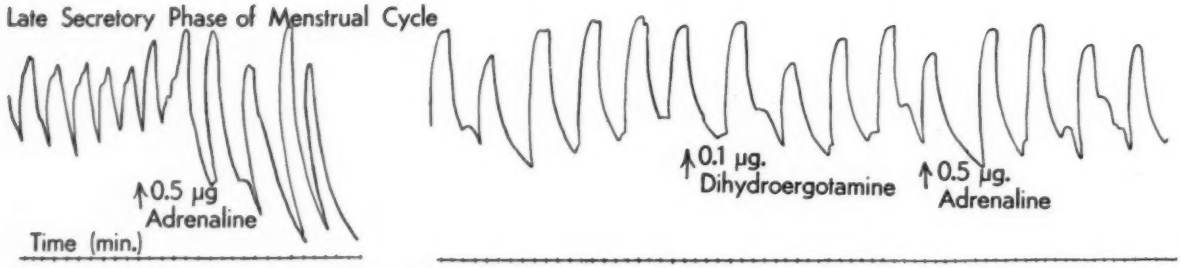


FIG. 1.—Non-pregnant myometrium in the late secretory phase of the menstrual cycle. Each dose on tracing is the final concn./ml. Adrenaline 1: 2,000,000 causes augmentation of the established activity. In the same specimen dihydroergotamine 1: 10,000,000 blocks the response to adrenaline.

the specimens were set up in the apparatus. Rather than initiate contractions by chemical or mechanical stimuli, records were not begun until this latent period had passed and the spontaneous rhythm was well established and constant.

## RESULTS

The latent period before spontaneous rhythm was established varied with the physiological state of the material. Post-menopausal myometrium exhibited regular rhythm half to one hour after suspension in the bath; myometrium obtained during menstrual life began to contract in 2 to 3 hr., whereas specimens from late pregnancy and labour frequently failed to show any spontaneous activity at all, if examined within 24 hr. of removal. These pregnant specimens, however, if kept 24 to 48 hr. after their removal at caesarean section, showed contractions 2 to 3 hr. after being set up in the bath.

Failure to wait for this latent period to pass, together with the use of normal saline in place of Ringer-Locke's solution, doubtless explains Goldfarb's (1954) unusual results with pituitary extract on excised myometrium in late pregnancy.

### *Adrenaline and Noradrenaline*

### *Non-pregnant Uterus*

*Before the Menopause.*—Both adrenaline (as hydrochloride) and noradrenaline (as bitartrate) stimulated the non-pregnant human myometrium *in vitro*. With low concentrations of the active bases (e.g. up to 1 in 2,000,000), augmentation of established activity was observed (Fig. 1), but with higher concentrations (1 in 1,000,000 to 1 in 200,000) the muscle went into spasm, which persisted for some minutes (Fig. 2).

This reaction was obtained with adrenaline in 15 strips from 17 uteri. Doses well below the threshold concentrations produced no reaction in the remaining 2 specimens (Table I).

Stimulation was similarly observed with noradrenaline in strips from 17 out of 19 non-pregnant uteri. Subthreshold concentrations accounted for one of the specimens failing to respond. In the other specimen no reaction occurred with concentrations up to 1 in 200,000; with the excessively high concentration of 1 in 20,000, there was inhibition (Table I).

*After the Menopause.*—Similar responses to adrenaline and noradrenaline were obtained with the 8 strips examined from 5 post-menopausal uteri (Fig. 3).

A comparison, by the method of Litchfield and Wilcoxon (1949), of the concentrations of adrenaline and noradrenaline producing stimulation in 50% of the specimens (the ED50) showed that non-pregnant myometrium is 2½ times more sensitive to noradrenaline than to adrenaline.

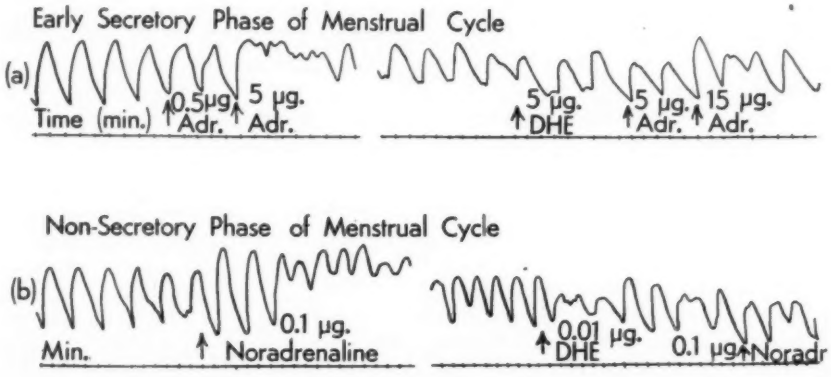


FIG. 2 (*a*) and (*b*).— Non-pregnant myometrium. Both adrenaline and noradrenaline stimulate the contracting myometrium. This response is blocked by dihydroergotamine. Each dose on tracing is the final concn./ml.

For all non-pregnant specimens the ED50 (and 19/20 confidence limits) for adrenaline was 1.38 (0.72 to 2.65) μg./ml., and for noradrenaline was 0.39 (0.21 to 0.71) μg./ml. The potency ratio is, therefore, $\frac{\text{Noradrenaline ED50}}{\text{Adrenaline ED50}}$ (and 19/20 confidence limits)=2.4 (1.5 to 8.5).

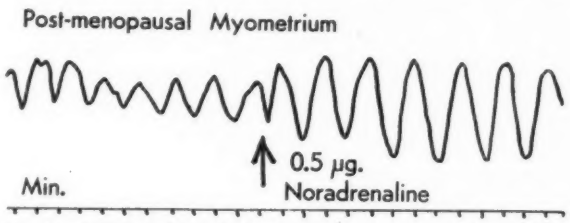


FIG. 3.—Augmentation of the spontaneous contractions of post-menopausal myometrium produced by noradrenaline 1 : 2,000,000. Dose on tracing is the final concn./ml.

Although differences were observed between the responses of specimens obtained during the endometrial, proliferative, and secretory phases of the menstrual cycle, these apparent variations were not statistically significant.

*Pregnant Uterus*

*Early Pregnancy.*—One specimen from the first trimester was obtained at hysterotomy from a

TABLE I

EFFECTS OF ADRENALINE AND OF NORADRENALINE ON STRIPS FROM INDIVIDUAL NON-PREGNANT UTERI

| Phase | Case No. | Adrenaline (μg./ml.) | | | | | | | Noradrenaline (μg./ml.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0·001 | 0·01 | 0·1 | 0·5 | 1 | 5 | 20 | 0·001 | 0·01 | 0·1 | 0·5 | 1 | 5 | 20 | 50 |
| Proliferative .. | 1 | | | 0 | | | | | | | 0 | | | | | |
| | 2 | | | | 0 | 0 | + | | | | | | 0 | + | | |
| | 3 | | | | | | | | | | + | + | | + | | |
| | 4 | | | 0 | 0 | | | | | | | | | | | |
| | 5 | | | | | | | | | | 0 | 0 | | + | | |
| | 6 | | | 0 | 0 | | + | | | | 0 | + | | + | | |
| | 7 | | | | 0 | | 0 | + | | | | 0 | | | + | |
| | 8 | | | | | | | | | | | 0 | 0 | ± | + | |
| | 9 | | | | | 0 | + | | | | + | | | + | | |
| | 10 | | | | 0 | | + | | | | + | | + | + | | |
| | 11 | | | | | | | | | | 0 | | 0 | 0 | | − |
| | 12 | | | | | | | | | | | | | + | | |
| Secretory .. | 13 | 0 | 0 | 0 | + | | | | 0 | 0 | 0 | + | + | | | |
| | 14 | | | | 0 | 0 | + | + | | | | | | | | |
| | 15 | | | | | + | | | | | | | + | | | |
| | 16 | | 0 | 0 | + | + | | | | | 0 | + | | | | |
| | 17 | | | | 0 | 0 | + | | | | | | | | | |
| | 18 | | | | 0 | 0 | 0 | + | | | | 0 | | + | | |
| | 19 | | | | | | + | | | | | | | + | | |
| | 20 | | | | + | | | | | | + | | | | | |
| | 21 | | | | 0 | | + | | | | | + | | | | |
| | 22 | | | | + | | | | | | | | 0 | | + | |
| Post-menopausal | 23 | | | | 0 | 0 | + | | | | | + | | | | |
| | 24 | | | | | | + | | | | | | | + | | |
| | 25 | | 0 | 0 | 0 | | + | | | | + | + | + | | | |
| | 26 | | | | | | + | | | | | | | + | | |
| | 27 | | | | 0 | | 0 | | | | | 0 | | + | | |

0=No effect. +=Stimulation. ±=Equivocal response. −=Inhibition.

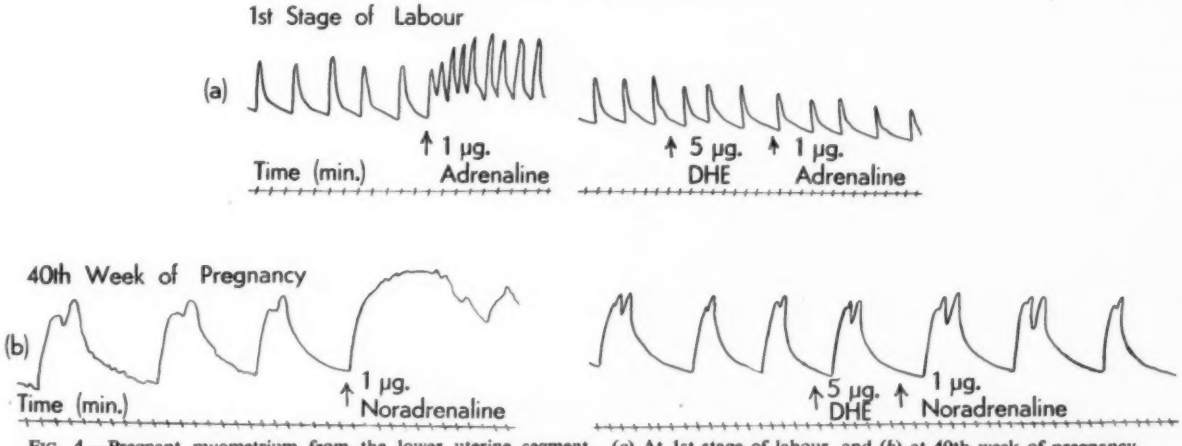


FIG. 4.—Pregnant myometrium from the lower uterine segment. (*a*) At 1st stage of labour, and (*b*) at 40th week of pregnancy. Each dose on tracing is the final concn./ml. Both adrenaline and noradrenaline stimulate the contracting myometrium. This response is blocked by dihydroergotamine.

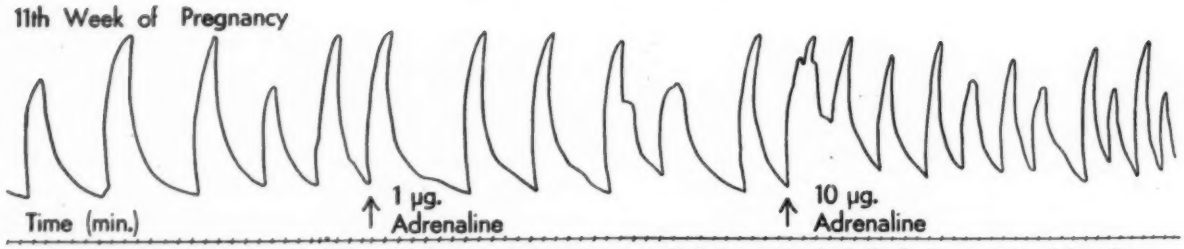


FIG. 5.—Myometrium from the body of the uterus in the first trimester. Each dose on tracing is the final concn./ml. Adrenaline produces an equivocal result: in concentration of 1: 100,000 there is increased frequency but decreased amplitude of contractions with slight rise in base line.

patient who gave a history of 11 weeks' amenorrhoea. Myometrial strips from this case were stimulated by noradrenaline, but gave equivocal results with adrenaline. The latter caused increased frequency of contraction with some raising of the base line, but with decreased amplitude (Fig. 5).

*Late Pregnancy and Labour.*—In the third trimester both adrenaline and noradrenaline stimulated the myometrium to contract. This reaction was obtained in strips from 9 out of 10 uteri with adrenaline, and with strips from all 10 uteri with noradrenaline (Table II).

These results with pregnant specimens from the lower uterine segment cannot be quantitatively compared with the other specimens in the whole series, as the non-pregnant specimens came from the body of the uterus. Gunn and Scott Russell (1946) showed that muscle from the upper segment is less sensitive to adrenaline than that from the lower segment, although their responses are qualitatively similar.

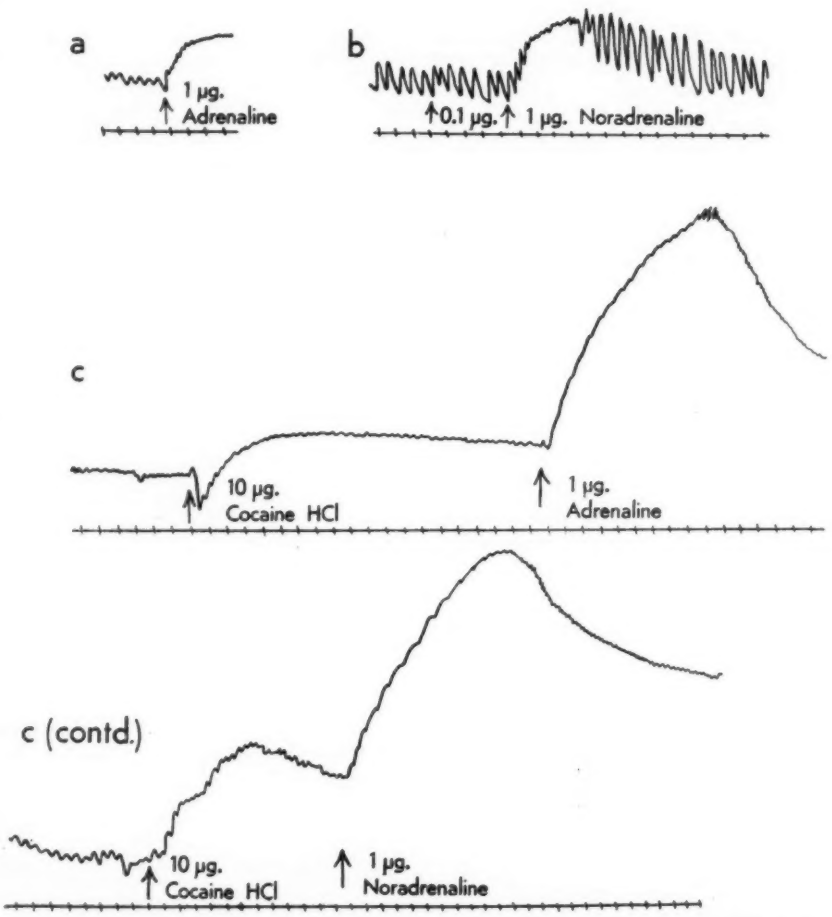


FIG. 6.—Three strips of myometrium from the same uterus at the 39th week of gestation. Each dose on tracing is the final concn./ml. In *a* and *b* adrenaline and noradrenaline produce stimulation. In *c* stimulation is also seen with these drugs in the presence of cocaine HCl 1: 100,000. Time in min.

The usual stimulation observed with adrenaline and noradrenaline in strips from both pregnant and non-pregnant uteri persisted after the addition of cocaine hydrochloride to the bath (Fig. 6).

### *Dihydroergotamine*

Dihydroergotamine itself has usually no direct action on spontaneously contracting myometrium *in vitro*. Occasionally it has inhibited contractions in non-pregnant specimens and stimulated contractions in a few pregnant specimens, but such responses are the exception, not the rule.

In 7 out of 10 non-pregnant uteri, and in all 6 pregnant uteri examined, the usual stimulating action of adrenaline was not seen after the addition of dihydroergotamine to the bath (Figs. 1, 2a, and 4a). In fact, its action was occasionally

TABLE II

EFFECTS OF ADRENALINE AND OF NORADRENALINE ON STRIPS FROM INDIVIDUAL PREGNANT UTERI

| Phase | Case No. | Adrenaline (μg./ml.) | | | | | Noradrenaline (μg./ml.) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0·1 | 0·5 | 1 | 5 | 10 | 0·1 | 0·5 | 1 | 5 |
| Early pregnancy | 28 | | | 0 | | ± | | + | + | + |
| Late pregnancy and labour | 29 | | | | + | | 0 | 0 | | + |
| | 30 | 0 | 0 | + | | | | | | |
| | 31 | + | | | | | 0 | 0 | | + |
| | 32 | | 0 | | 0 | 0 | | + | + | |
| | 33 | | 0 | | + | | | 0 | | + |
| | 34 | | | | + | | | | | + |
| | 35 | | | | | | | | | + |
| | 36 | | | | + | | | | | + |
| | 37 | + | | + | | | 0 | | + | |
| | 38 | | | 0 | | + | | | + | |
| | 39 | | | | | | | | | + |
| | 40 | | | + | | | | | | |

0 = No effect. + = Stimulation. ± = Equivocal response.

TABLE III

DETAILS OF EXPERIMENTS DEMONSTRATING THE ADRENALINE-BLOCKING PROPERTIES OF DIHYDRO-ERGOTAMINE

| Phase | Case No. | Stimulating Concn. of Adrenaline/ (μg./ml.) | Concn. of DHE (μg./ml.) | Repeated Concn. of Adrenaline (μg./ml.) | Ratio: Repeated Concn. Adrenaline/ Concn. DHE | Result* |
|---|---|---|---|---|---|---|
| Non-pregnant | 22 | 0·5 | 0·5 | 0·5 | 1 | − |
| | 9a | 10 | 10 | 10 | 1 | 0 |
| | 9b | 10 | 20 | 20 | 1 | − |
| | 21a | 5 | 5 | 5 | 1 | 0 |
| | 16a | 1 | 0·5 | 1 | 2 | 0 |
| | 14 | 20 | 10 | 20 | 2 | + |
| | 18 | 20 | 5 | 20 | 4 | − |
| | 21b | 5 | 5 | 20 | 4 | 0 |
| | 20 | 0·5 | 0·1 | 0·5 | 5 | 0 |
| | 16b | 1 | 0·5 | 5 | 10 | 0 |
| | 6a | 5 | 0·5 | 5 | 10 | 0 |
| | 6b | 5 | 0·5 | 10 | 20 | 0 |
| | 25 | 5 | 0·1 | 5 | 50 | + |
| | 23 | 5 | 0·1 | 5 | 500 | + |
| Pregnant | 40b | 1 | 5 | 1 | 0·2 | 0 |
| | 36 | 5 | 10 | 5 | 0·5 | 0 |
| | 31 | 0·1 | 0·1 | 0·1 | 1 | 0 |
| | 40a | 1 | 1 | 1 | 1 | +(much reduced) |
| | 33 | 5 | 5 | 5 | 1 | 0 |
| | 34a | 5 | 5 | 5 | 1 | 0 |
| | 34b | 5 | 5 | 20 | 4 | − |
| | 30a | 1 | 0·1 | 1 | 10 | 0 |
| | 30b | 1 | 0·1 | 5 | 50 | 0 |

* 0 = No effect. + = Stimulation. − = Inhibition.

reversed, and definite inhibition was observed (Table III).

Similarly, dihydroergotamine blocked the stimulating action of noradrenaline in 8 out of 11 non-pregnant uteri and in all 6 pregnant uteri examined (Figs. 2b and 4b ; Table IV).

## DISCUSSION

It has been shown that the spontaneous contractions of isolated human myometrium are stimulated by both adrenaline and noradrenaline, and that this reaction is qualitatively the same whether the specimen is obtained during one of the various stages of the menstrual cycle, after the menopause, during pregnancy or at parturition.

TABLE IV

DETAILS OF EXPERIMENTS DEMONSTRATING THE NORADRENALINE-BLOCKING PROPERTIES OF DIHYDRO-ERGOTAMINE

| Phase | Case No. | Stimulating Concn. of Noradrenaline (μg./ml.) | Concn. of DHE (μg./ml.) | Repeated Concn. of Noradrenaline (μg./ml.) | Ratio: Repeated Concn. Noradrenaline/ Concn. DHE | Result* |
|---|---|---|---|---|---|---|
| Non-pregnant | 21a | 0·5 | 1 | 0·5 | 0·5 | 0 |
| | 27 | 5 | 10 | 5 | 0·5 | 0 |
| | 20a | 0·1 | 0·1 | 0·1 | 1 | 0 |
| | 16a | 0·5 | 0·5 | 0·5 | 1 | 0 |
| | 9a | 2 | 2 | 2 | 1 | 0 |
| | 18 | 5 | 5 | 5 | 1 | 0 |
| | 16b | 0·5 | 0·5 | 1 | 2 | 0 |
| | 21b | 0·5 | 1 | 2 | 2 | 0 |
| | 20b | 0·1 | 0·1 | 0·5 | 5 | − |
| | 21c | 0·5 | 1 | 5 | 5 | 0 |
| | 9b | 2 | 2 | 10 | 5 | 0 |
| | 3a | 0·1 | 0·01 | 0·1 | 10 | 0 |
| | 25a | 0·1 | 0·01 | 0·1 | 10 | + |
| | 6a | 5 | 0·5 | 5 | 10 | + (reduced) |
| | 6b | 5 | 0·5 | 15 | 30 | + |
| | 3b | 0·1 | 0·01 | 0·5 | 50 | 0 |
| | 23 | 0·5 | 0·01 | 0·5 | 50 | + |
| | 25b | 0·5 | 0·1 | 5 | 50 | + |
| | 5 | 5 | 0·1 | 5 | 50 | 0 |
| Pregnant | 37b | 1 | 25 | 1 | 0·04 | 0 |
| | 37a | 1 | 10 | 1 | 0·1 | + (reduced) |
| | 37c | 1 | 25 | 5 | 0·2 | +(much reduced) |
| | 38 | 1 | 5 | 1 | 0·2 | 0 |
| | 35b | 5 | 20 | 5 | 0·25 | 0 |
| | 36 | 5 | 10 | 5 | 0·5 | 0 |
| | 35a | 5 | 5 | 5 | 1 | +(much reduced) |
| | 33a | 5 | 5 | 5 | 1 | 0 |
| | 33b | 5 | 5 | 15 | 3 | 0 |
| | 31 | 5 | 0·1 | 5 | 50 | 0 |

* 0 = No effect. + = Stimulation. − = Inhibition.

The site of action of adrenaline and noradrenaline is probably on the plain muscle cells themselves rather than on the nervous elements included in the myometrial strip. When the nerve endings are paralysed by cocaine the responses to adrenaline and to noradrenaline persist.

From the point of view of comparative physiology, the human uterus must then be placed in the " rabbit group " of Greef and Holtz's table ; this includes the animals in which adrenaline and noradrenaline stimulate both the pregnant and non-pregnant uterus. As all of this table is based on evidence from studies *in vitro*, this is justifiable, but there are certain other factors which will now be considered.

Kaiser (1951) showed, and Garrett (1954) confirmed more directly, that adrenaline *inhibits* the

contractions of the intact human uterus in late pregnancy and labour, whereas noradrenaline stimulates them. As far as adrenaline is concerned this would appear to be a direct conflict of results, yet each of these conclusions has been drawn from experiments in which the results have always proved consistent. It may be said that *in vitro* methods are so unnatural as to produce spurious responses, but the results have invariably been the same. If they are to be accepted as being indicative of the physiological processes involved in uterine contraction, we are faced with a seeming conundrum. Perhaps the contractions measured *in vitro* are a different entity altogether from the large co-ordinated contractions of the whole uterus observed in the patient in late pregnancy and parturition. If so, further work is necessary to produce a satisfactory synthesis of the results of experiments to date.

Just as Orth and Ritchie (1947) have shown in the heart, and Greef and Holtz (1951a) have shown in the uterus of lower mammals, that dihydroergotamine can block the characteristic response to adrenaline and noradrenaline, so too in this present work has this been shown to be true of excised human uterine muscle. However, these adrenaline-blocking properties have yet to be demonstrated *in vivo*; the writer is studying this problem at present.

## Summary

1. Experiments *in vitro* with strips of myometrium from 40 human uteri are described.

2. Both adrenaline and noradrenaline stimulate the spontaneous contractions of human myometrium obtained at the various stages of the menstrual cycle, after the menopause, during pregnancy and at parturition.

3. Noradrenaline has a greater stimulant action on non-pregnant myometrium than has adrenaline.

4. The stimulant action of adrenaline and noradrenaline on human myometrium persists in the presence of cocaine.

5. Dihydroergotamine blocks the stimulant action of adrenaline and noradrenaline on human myometrium *in vitro*.

6. These *in vitro* results are compared with the known actions of adrenaline and noradrenaline on the intact human uterus in late pregnancy and parturition. The *in vitro* and *in vivo* results differ in certain essential respects.

This work was done in the Nuffield Department of Obstetrics and Gynaecology, Oxford, under the supervision of Professor J. Chassar Moir. My thanks are also due to Professor J. H. Burn and Emeritus Professor J. A. Gunn for their many useful suggestions in this work and for their valuable criticism.

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# THE EFFECT OF NALORPHINE ON THE ANTIDIURETIC ACTION OF MORPHINE IN RATS AND MEN

BY

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In several mammalian species the diuretic response to water given by mouth is inhibited by morphine. De Bodo (1944) concluded, from experiments on dogs, that this inhibition cannot be attributed to the effect of morphine on water absorption from the gut; he suggested that it was due to release of posterior pituitary antidiuretic hormone.

Nalorphine (*N*-allylnormorphine) antagonizes some of the pharmacological actions of morphine —for example, its effect on respiration (McCawley, Hart, and Marsh, 1941; Bodman, 1953) and its analgesic action (Unna, 1943; Hart and McCawley, 1944). It seemed of interest, therefore, to see whether nalorphine would antagonize the antidiuretic effect of morphine, and whether this could be achieved without affecting its analgesic action.

## METHODS

Effects on renal water excretion in rats were tested by a method similar to that described by Ginsburg (1951). Adult male albino rats weighing approximately 200 g. were used. They were deprived of food for 16 hr. before the test, but were allowed free access to water. Each animal was then weighed, given water equivalent to 5% of its body wt. by stomach tube and placed in a metabolism cage. One hr. later the animals were injected subcutaneously with the drug or drugs to be tested, and were again given water equivalent to 5% of their body wt. The volumes of urine excreted in the first hr. were noted. The "water load" of the animal at the beginning of the second hr. could thus be calculated. Urine volumes were noted at 30 min. intervals for the following 3 hr. Immediately before each reading the animals were made to empty their bladders, by prodding or stroking. Cross-over experiments were done with at least a week's interval between tests. Morphine sulphate and nalorphine hydrobromide dissolved in 0.9% NaCl solution were used. Intestinal water absorption was measured by the method of Heller and Smirk (1932). Analgesic effects in rats were estimated by the time required from exposure to a standard thermal stimulus until the rat moves its tail (Davies, Raventos, and Walpole, 1946). To avoid injury the animal's tail was never exposed to the stimulus for more than 20 sec.

The water content of the rat brains was determined as follows. Two groups of adult male rats of 180–210 g. were treated as in the diuresis tests. One group was injected with 0.9% NaCl solution and the other with morphine sulphate (10 mg./kg.). At 0, 90, 180, and 270 min. after the injection, rats from both groups were quickly anaesthetized with ether and killed by bleeding. The brain was carefully dissected, placed on a weighed watchglass, and weighed. The cerebellum, cerebral hemispheres, medulla, and pons were immediately dissected from the basal nuclei and each of these pieces of brain weighed separately. The dissected brains were then dried.

Water diuresis in man was investigated as follows: The subjects were deprived of food and fluids for 12 hr. before the test. They then emptied their bladders and had the drug or drugs injected intramuscularly. Immediately afterwards they drank a standard volume of water (860 ml./m.$^2$ body surface). They lay on couches during the test, but were allowed to stand up to pass urine. In one instance a subject was not allowed to sit or stand during the test. Urine was collected at hourly intervals. Whenever possible cross-over experiments were performed with at least a week's interval between tests. All volunteers were unaware of what drug or drugs they received, and the drugs were given in a random manner.

## RESULTS

*Antidiuresis in Rats.*—Fig. 1 shows the marked antidiuretic effect of a subcutaneous injection of 10 mg./kg. morphine sulphate in rats. When this dose of morphine sulphate (=MO) was injected simultaneously with nalorphine hydrobromide (=NA), in the ratio of 1:2.5, there was a marked diminution in the antidiuretic response. When, however, the procedure was repeated but the ratio MO:NA was 12:1 there was no significant difference in the antidiuretic response. When the ratio MO:NA was 6:1 there was a slight diminution in the antidiuretic effect: rats, given morphine

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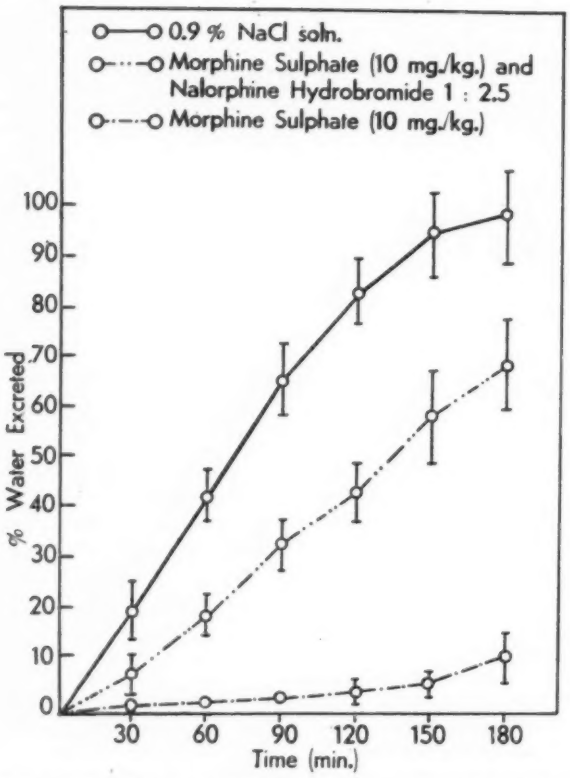


FIG. 1.—The effect on the antidiuretic action of morphine sulphate in rats of the simultaneous injection of nalorphine hydrobromide and morphine sulphate in the dose ratio MO: NA = 1: 2.5.

(10 mg./kg.) and nalorphine simultaneously in the ratio of 12:1, excreted 24.7 ± 1.4% (mean ± S.E.) of the water load in 3 hr., whereas when the ratio was 6:1 the excretion was 41.2 ± 4.7% in a similar time. Rats given morphine sulphate only, excreted 23.8 ± 0.8% of their water load in 3 hr.; the control animals injected with 0.9% NaCl solution excreted 92.1 ± 9.1% of their water load during the same period.

Similar experiments were done to see whether nalorphine by itself had any antidiuretic effects in rats. There was no significant difference between rats given nalorphine hydrobromide (0.8 mg./kg. or 25 mg./kg.) subcutaneously and controls given 0.9% NaCl solution. The rats given nalorphine excreted 96.0 ± 11.2% (mean ± S.E.) of the water load in 3 hr. as against 99.9 ± 6.6% ($t = 0.56$ $P > 0.5$) in the controls. Rats given 25 mg./kg. nalorphine HBr excreted 90.2 ± 8.2%, and their controls excreted 99.0 ± 7.6% ($t = 0.88$ $P > 0.4$).

Table I shows the effect of morphine, nalorphine, and of the two drugs combined, on gastro-intestinal water absorption 180 min. after injection. There was a significant difference between the mean weight of the gastro-intestinal tracts of the group of rats injected with morphine and that of the control group. This was mainly due to differences in stomach weight. Pyloric spasm was seen in many of the morphine-treated animals when the abdomen was opened 180 min. after the administration of water. Similar results were obtained 90 min. after the administration of water and drug. However, even if both the increase in gastric emptying time and the decrease in the rate of intestinal water absorption are taken into account, and urine volume is expressed as % of absorbed water excreted, rats which received morphine sulphate and nalorphine hydrobromide in a dose ratio of 1:2.5 excreted more of the administered water than the animals which received morphine only (Fig. 2).

*Analgesia in Rats.*—Table II shows the reaction time of rats to a standard thermal stimulus at various intervals after the injection of nalorphine, morphine, or a combination of these drugs. The rats given 0.9% NaCl solution maintained much the same reaction time (8–10 sec.) during the course of the test; animals given morphine showed marked analgesia (reaction time uniformly >20 sec.); nalorphine (25 mg./kg.) alone had a slight analgesic effect most marked after about 40 min. Although analgesic effects could also be demon-

TABLE I

THE EFFECT OF NALORPHINE, OF MORPHINE, AND OF MORPHINE AND NALORPHINE SIMULTANEOUSLY, ON THE WT. OF THE RAT'S INTESTINAL TRACT AFTER ADMINISTRATION OF WATER

(The numerals are the means ± S.E. from groups of 6 animals. Numerals in parentheses are % changes)

| Treatment | After 90 min. | | | After 180 min. | | |
|---|---|---|---|---|---|---|
| | Wt. of Stomach (g.) | Wt. of Intestine (g.) | Wt. of Whole Gastro-intestinal Tract (g.) | Wt. of Stomach (g.) | Wt. of Intestine (g.) | Wt. of Whole Gastro-intestinal Tract (g.) |
| 0·9% NaCl soln. .. .. | 5·9 ± 0·1 | 10·2 ± 0·1 | 16·1 ± 0·4 | 5·9 ± 0·1 | 10·2 ± 0·1 | 16·1 ± 0·1 |
| Morphine sulphate, 10 mg./kg. | 7·1 ± 0·2 (+20) | 10·5 ± 0·6 (+3) | 17·6 ± 0·7 (+9) | 6·9 ± 0·1 (+17) | 10·9 ± 0·2 (+7) | 17·8 ± 0·2 (+11) |
| Nalorphine hydrobromide, 25 mg./kg. | 6·5 ± 0·1 (+10) | 10·5 ± 0·1 (+3) | 17·0 ± 0·1 (+6) | 6·0 ± 0·1 (+2) | 10·1 ± 0·3 (−1) | 16·2 ± 0·4 (+1) |
| Morphine sulphate: nalorphine hydrobromide, 1:2·5 | 6·5 ± 0·2 (+10) | 10·4 ± 0·1 (+2) | 17·0 ± 0·5 (+6) | 6·4 ± 0·1 (+9) | 10·0 ± 0·1 (−2) | 16·4 ± 0·1 (+2) |

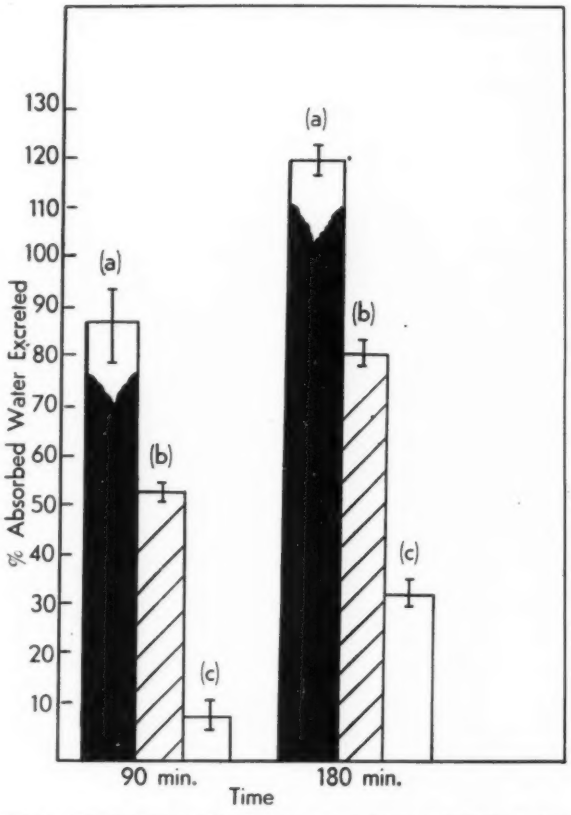


FIG. 2.—Comparison of the effect of subcutaneous nalorphine hydrobromide and morphine sulphate given simultaneously, with that of morphine sulphate alone, on the percentage of absorbed water excreted 90 min. and 180 min. after administration of the water. (*a*) 0.9% NaCl soln.; (*b*) morphine sulphate (10 mg./kg.): nalorphine hydrobromide, 1: 2.5; (*c*) morphine sulphate (10 mg./kg.).

TABLE II

REACTION TIMES (SEC.) OF RATS TO A STANDARD THERMAL STIMULUS BEFORE AND AFTER VARIOUS TREATMENTS

(All rats were given 10% of their body weight of water in two divided doses in addition to the NaCl soln. or the drugs. The numerals are the means ± S.E. Numerals in parentheses are no. of animals per group.)

| Treatment | Before Injection | Min. after Injection | | |
|---|---|---|---|---|
| | | 40 | 80 | 155 |
| NaCl 0·9% soln. .. | 9·6 ± 0·6 (11) | 9·8 ± 0·7 (10) | 7·6 ± 0·6 (10) | 9·1 ± 0·9 (7) |
| Morphine sulphate 10 mg./kg. | 7·6 ± 0·7 (11) | All > 20 (10) | All > 20 (10) | All > 20 (7) |
| Nalorphine hydrobromide, 25 mg./kg. | 10·4 ± 1 (10) | 10, > 20, 9, 12, 13, 10, 19, 17, > 20, 13* | 12·1 ± 1·3 (10) | 7, 15, 10, 9, 12, > 20, 11* |
| Morphine sulphate: nalorphine hydrobromide 1: 2·5 | 10·9 ± 0·9 (10) | 18 > 20, 11, > 20, 14, 11, 5, 20, 17, 16* | > 20, 14, > 20, 11, 7, 13, 8, 10, 17* | > 20, 9, > 20, > 20, 12, 16, 12* |

* Reaction times of individual rats.

strated in animals given morphine sulphate and nalorphine hydrobromide, the morphine analgesia was diminished by the nalorphine. The dose of nalorphine which diminishes the antidiuretic effect of morphine in rats also, therefore, weakens its analgesic action.

*Antidiuresis in Man.*—The effects of morphine, nalorphine and combinations of these drugs on water diuresis were also tested in healthy men aged from 19–35. Nalorphine alone (9.2 mg./m.²) was given to 5 subjects. The results were compared with those of control experiments in which, a week before or after the experiment, the subject received an injection of 0.9% NaCl solution. Two types of response to nalorphine were observed: three men showed no inhibition of their renal response to water, but two showed a pronounced inhibition of water diuresis (Fig. 3). The inhibition could not be correlated with clinical signs and symptoms, for nalorphine, in the dose used, produced pallor, giddiness, mild euphoria, and disorientation in all the subjects.

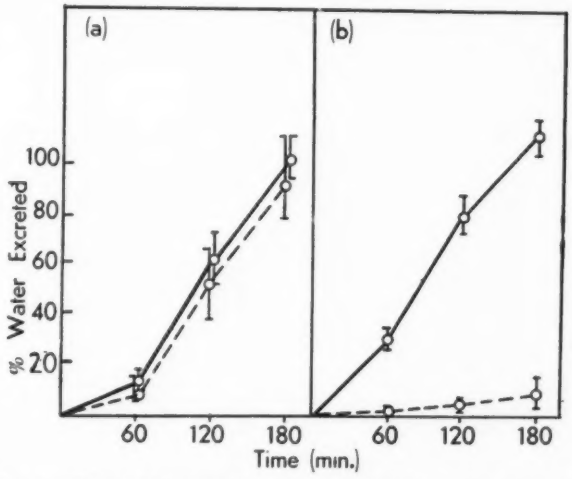


FIG. 3.—Effect of intramuscular nalorphine hydrobromide (9.2 mg./m.²) on the water diuresis of healthy human subjects. (*a*) Mean results in 3 subjects; (*b*) mean results in 2 subjects. Continuous line, 0.9% NaCl solution. Broken line, nalorphine hydrobromide, 9.2 mg./m.²

Morphine sulphate (9.2 mg./m.²) produced a variable degree of inhibition of water diuresis in all the subjects investigated. After morphine, five subjects had excreted 33.4 ± 13.7% (mean ± S.E.) of the water load after 3 hr., whereas after normal saline the same subjects had excreted over 100% (105.4 ± 0.4) of the water load (Fig. 4). In one subject, given morphine sulphate (9.2 mg./m.²) 45 min. after administration of water, and not allowed to get up during the test, inhibition of

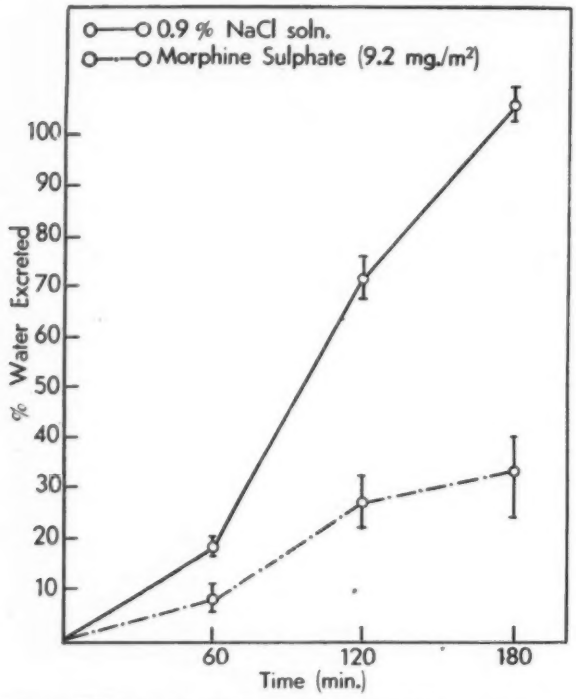


FIG. 4.—Effect of 9.2 mg./m.² morphine sulphate on the water diuresis of 5 healthy men.

water diuresis was also observed. He excreted 7% of his water load in 3 hr. after morphine, compared with 92% after 0.9% NaCl solution.

*Side Effects in Man.*—The toxic side effects of morphine seemed to be delayed by the administration of the water load. All subjects were drowsy and one subject vomited. The drowsiness became more marked between 3 and 9 hr. after injection, and several subjects vomited frequently. One man developed marked respiratory depression during this period. The subjects who showed the most marked delay in water excretion also exhibited the most marked toxic symptoms between the 3rd and 9th hours. Further tests on human volunteers thus seemed inadvisable. In contrast, however, to the results in rats, nalorphine and morphine in the dose ratio 1:1 did not increase the renal response of the subjects to water (Table III).

In similar experiments using morphine combined with nalorphine, in the ratio 1:2.5 (Table III), antidiuretic effects were still observed. These subjects showed toxic effects such as giddiness, euphoria and nausea.

It was thought that the delayed toxic effects of morphine were due to cerebral oedema. To test this hypothesis the effect of morphine sulphate on the water content of the brains of rats was investigated.

TABLE III

HEALTHY MEN. EFFECT ON WATER DIURESIS OF COMBINED TREATMENT WITH MORPHINE SULPHATE (MO) (9·2 MG./M.²) AND NALORPHINE HYDROBROMIDE (NA), COMPARED WITH MORPHINE SULPHATE ALONE

| Subject and Age (yr.) | Body Weight (kg.) | Surface Area (m.²) | Drug and Dose (mg.) | % Water Load Excreted in 3 hr. |
|---|---|---|---|---|
| | A. Dose ratio MO: NA = 1:1 | | | |
| R.B. (27) | 63 | 1·75 | MO 16·1<br>NA 16·1 | 34 |
| R.B. (27) | 63 | 1·75 | MO 16·1 | 29 |
| C.W. (34) | 95 | 2·15 | MO 19·8<br>NA 19·8 | 25 |
| C.W. (34) | 95 | 2·15 | MO 19·8 | 63 |
| J.M. (35) | 70·2 | 1·81 | MO 16·7<br>NA 16·7 | 15 |
| J.M. (35) | 70·2 | 1·81 | MO 16·7 | 46 |
| | B. Dose ratio MO: NA = 1:2·5 | | | |
| E.J. (19) | 66·5 | 1·75 | MO 16·1<br>NA 40·3 | 2·0 |
| E.S. (19) | 71·4 | 1·8 | MO 16·6<br>NA 41·5 | 24·0 |
| R.K. (23) | 65·4 | 1·75 | MO 16·1<br>NA 40·3 | 81·0 |
| J.A. (23) | 65·2 | 1·78 | MO 16·4 | 78·0 |
| H.S. (27) | 65·4 | 1·85 | 0·9% NaCl | 107·0 |

Table IV shows the water content of the brains 0, 90, 180 and 270 min. after the injection of 10 mg./kg. morphine sulphate. No significant difference from the controls could be detected. The same method gave similarly negative results when the water content of the cerebral hemispheres, basal nuclei, medulla and pons, and cerebellum were estimated separately.

TABLE IV

EFFECT OF MORPHINE SULPHATE ON THE BRAIN WATER CONTENT OF HYDRATED RATS (EXPRESSED AS % WT. OF BRAIN)

(Mean ± standard error from groups of 5 animals)

| Time after Injection (min.) | NaCl Soln. (0·9%) | Morphine Sulphate (10 mg./kg.) |
|---|---|---|
| 0 | 44·2 ± 1·6 | 43·8 ± 1·4 |
| 90 | 43·8 ± 1·4 | 42·8 ± 1·7 |
| 180 | 44·6 ± 0·2 | 44·3 ± 1·2 |
| 270 | 43·4 ± 1·9 | 41·4 ± 1·0 |

## DISCUSSION

Nalorphine may decrease the antidiuretic effect of morphine in several ways. An action of morphine on the posterior pituitary is suggested by the fact that, in dogs, direct injection of morphine into the supra optic nucleus produces an antidiuresis (Duke, Pickford, and Watt, 1951), and that morphine does not inhibit urine flow in "neurohypophysectomized" animals (De Bodo, 1944). Large doses, however (2 mg./kg. i.v.), also influence glomerular filtration rate (Handley and Keller, 1950). Similarly Giarman, Mattie and Stephenson (1953) found more antidiuretic

activity in the urine of intact rats given morphine than in hypophysectomized animals. Lipschitz and Stokey (1947), on the other hand, reported that renal denervation lessened the antidiuretic effect of morphine. It seems possible, therefore, that nalorphine prevents, or decreases, the release of the pituitary antidiuretic principle by morphine in the rat. However, since no measurements of renal plasma flow and glomerular filtration rate were done, another and perhaps additional mechanism—namely, antagonism to the renal vascular effects of morphine—cannot be excluded.

De Bodo (1944) found no impairment of water absorption from the gastro-intestinal tract in dogs given morphine. This seems, at first sight, to be at variance with our results. Apart from the difference in species, however, de Bodo injected the morphine 40 min. after the administration of water, whereas in our experiments on rats the morphine and water were given simultaneously. Hart and McCawley (1944) showed that nalorphine (0.5 mg./kg.) decreased the effect of morphine (1.0 mg./kg.) on the tone of the gut in unanaesthetized dogs with Thiry Vella loops. Similar results were obtained in man (Beal and Shapiro, 1953) and in unanaesthetized dogs with modified Mann loops (Gruber and Gruber, 1953). The effect of nalorphine in decreasing the action of morphine on the water absorption in rats may thus be due to a gastro-intestinal effect.

Hart and McCawley (1944) believe that nalorphine is at least as potent as morphine in raising the pain threshold of rats. Unna (1943), in mice, and Winter, Orahovats, Flataker, Lehman, and Lehman (1954), in rats, concluded that the analgesic effect of nalorphine was much lower; our results agree with the latter conclusion.

The fact that nalorphine in a dose which produced a moderate decrease in the antidiuretic effect of morphine in rats also weakened its analgesic effect, and that nalorphine in doses of 9.2 mg./m.$^2$ and 23 mg./m.$^2$ failed to have any appreciable effect on the antidiuretic action of morphine in the few human subjects used, indicate that it would be of little clinical use in preventing antidiuresis from morphine.

It is well known that the analgesic response to a standard dose of morphine varies considerably in human beings. Nalorphine has many of the actions of morphine though to a lesser degree. One would therefore expect that the individual response to nalorphine would vary similarly. Moreover, those subjects who did not show the antidiuretic effect of nalorphine after the administration of water would probably have done so if the dose had been increased. However, larger doses could not be given since, even with the dose used, all subjects showed mild toxic effects.

Whether morphine has an antidiuretic effect in human beings is still a controversial issue (Table V), but antidiuresis was seen in all of our experiments. It may have been due to diminution

TABLE V

EFFECT OF MORPHINE SULPHATE ON THE RENAL RESPONSE OF HUMAN BEINGS TO WATER

| Authors | Subjects | Posture | Dose and Route of Admin. | Renal Response to Water Load |
|---|---|---|---|---|
| Fee (1928) .. | Male adult | Not stated | 16 mg. s.c. | Diminished |
| Bahn, Iserbeck, and Linderman (1930) | Patients with sciatica and nephrolithiasis | ,, ,, | 20 ,, ,, | ,, |
| Ferrer and Sokaloff (1947) | Patients (convalescent) | ,, ,, | 10–16 mg. i.m. | ,, |
| Walker (1949) | Students | ,, ,, | 20 mg. s.c. | Unchanged |
| Lewis (1953) | Male Patient | Recumbent | 5·4 mg. i.v. | ,, |
| Schnieden and Blackmore (this paper) | Healthy male subjects | Standing Recumbent | 16·1 mg. i.m. | Diminished ,, |
| | | Standing | 16·1–18·4 mg. i.m. | ,, |

of renal blood flow or stimulation of the posterior pituitary. A decrease in urine flow still occurred in a subject kept horizontal, so standing cannot be an important factor. Decreased water absorption also can only be contributory, since morphine inhibited urine flow, even when 45 min. were allowed for water to be absorbed. Spurious antidiuresis due to action of morphine on the sphincter muscle of the bladder could also be excluded.

The ability of nalorphine to diminish the "antidiuretic" effect of morphine in man may be a question of the dose ratio. In rats, nalorphine HBr, administered with morphine sulphate in a dose ratio MO:NA=12:1, caused no difference in the renal response to water compared with animals given morphine only. When the dose ratio MO:NA was raised 1:2.5, the antidiuretic effect of morphine was markedly diminished. Since, however, the highest dose of nalorphine hydrobromide used in man (42 mg./m.$^2$) already produced slight toxic effects, it seemed undesirable to raise the dose further.

The delayed toxic effects of morphine sulphate (9.2 mg./m.$^2$) may conceivably have been due to the extra water load, but it should be pointed out that this was never greater than 2% of the body weight. Ellerbrook, Dunham, and Barbour (1930)

have shown that massive doses of vasopressin injected into hydrated rabbits caused oedema of the brain. It was therefore thought that the following sequence of events may have produced the toxic effects in human beings: stimulation by morphine of the neurohypophysis, increased release of antidiuretic hormone, and oedema of the brain leading to vomiting, drowsiness, and respiratory depression. There was, however, no difference between the water content of various parts of the brain in hydrated rats given morphine and that of control rats given 0.9% NaCl.

## Summary

1. The antidiuretic effect of morphine in rats is diminished by the simultaneous injection of a dose of nalorphine which alone produces no antidiuretic effect.

2. The effect of morphine is due partially to an action on gastric emptying time; simultaneous administration of nalorphine partially counteracts this effect. After water has been absorbed, morphine inhibits its excretion. Simultaneous administration of nalorphine diminishes this effect.

3. Nalorphine reduces the analgesic effect of morphine. Nalorphine itself produces slight analgesia.

4. The water content of the brain of rats to which water has been administered is not significantly higher after morphine than after saline.

5. Morphine has a marked antidiuretic effect on healthy men given water. Nalorphine produces an antidiuresis in some subjects.

6. Nalorphine injected simultaneously with morphine does not prevent the occurrence of marked antidiuretic effects in men.

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# HISTAMINE RELEASE AND THE "STRESS" PHENOMENON

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Various authors have shown that histamine causes depletion of the rat's adrenal ascorbic acid (Sayers and Sayers, 1947; Nasmyth, 1951; and Halpern and Benos, 1952), and in 1948 Ellinger suggested that released histamine might be a mediator of the "stress" phenomenon. Attempts to test this possibility (Tepperman, Rakieten, Birnie, and Diermeier, 1951) showed that both phenoxadrine and tripelennamine reduced the effect of histamine on the rat adrenal cortex, but not that of intraperitoneal carbon tetrachloride. They considered that they had no evidence either for or against the possibility that released histamine is concerned in the stress phenomenon. More recently, Nasmyth (1954) examined the effect of morphine, which produces the stress phenomenon, on the rat's adrenal, and concluded that, though both the adrenaline and the histamine which the drug releases affected the phenomenon, they were not wholly responsible for it.

These experiments were not conclusive, and it was felt that some useful information might be gained from an investigation of the adrenal response to a more potent histamine liberator than morphine, namely, compound 48/80, using depletion of the adrenal ascorbic acid as an index of cortical activity.

## METHODS

Except that only Wistar strain rats from Dr. Mandl's colony were used, and that 48/80 (0.5 mg./100 g. body wt.) replaced morphine, the methods (for the administration of drugs, the extraction of the adrenal glands, the extraction and estimation of skin histamine, the demedullation of the adrenals, and the depletion of skin histamine) were as described in a previous paper (Nasmyth, 1954).

Blood-pressure records were taken from the carotid artery of heparinized animals anaesthetized with urethane (7 ml./kg. of a 25% w/v solution), and were recorded by a Hg manometer similar to that of Condon (1951).

## RESULTS

*Effect of 48/80 in Normal Rats.*—The subcutaneous injection of 0.5 mg./100 g. body weight of 48/80 in normal rats caused prostration, cyanosis, oedema of the paws and snout, and itching. These signs persisted for 2–3 hr. and were accompanied by copious water drinking. Subsequently they lessened, and after 5 hr. the behaviour of the test animals was hardly distinguishable from that of the controls.

The changes in the adrenal content of ascorbic acid during the experiment reflected these events. Control animals, given a subcutaneous injection of normal saline, had an average adrenal ascorbic acid content of 330 mg./100 g. gland. One and a half hr. after the injection of 48/80 the content was 182 mg./100 g., or 55% of normal. It remained low, at 184 mg./100 g., 3 hr. after the 48/80; but after 5 hr. the recovery process had begun, and the mean value was 235 mg./100 g., or 71% of normal (Table I).

A substantial amount of histamine was released from the tissues of the test animals, as indicated by the mean values for the abdominal skin histamine. The average figure for the abdominal skin histamine of saline-injected controls was 58.1 μg./g. skin.* One and a half hr. after the injection of 48/80 the value was 22.9 μg./g. Three and five hours after the dose the figures were 19.2 μg./g. and 19.3 μg./g. respectively (Table I).

*Effect of 48/80 in Histamine-depleted Rats.*—An attempt was made to deplete the tissue histamine with propamidine, in doses of 0.4 to 2.0 mg., using a technique similar to that described for 48/80 (Nasmyth, 1954). As these doses did not cause any significant depletion of the skin histamine, and higher doses killed the animals, the

* This high value was obtained in animals which were used just after weaning. It originally gave some concern until Hardwick (1954) showed that at weaning there is a considerable but transient rise in the rat's skin histamine.

TABLE I

THE EFFECT OF COMPOUND 48/80 (0·5 MG./100 G.) ON THE ADRENAL ASCORBIC ACID OF RATS (CONTROL ANIMALS WERE GIVEN 0·9% SALINE, 0·25 ML./100 G.)

| Rats | Drug | Duration of Action (Hr.) | Ascorbic Acid Content (mg./100 g Gland ± S.E.) | Ascorbic Acid (% Normal) | No. of Glands | Abdominal Skin Histamine (μg. Base/g. Skin ± S.D.) |
|---|---|---|---|---|---|---|
| Normal | Saline | 1 to 3 | 330 ± 7 | 100 | 18 | 58·1 ± 16 |
| | 48/80 | 1·5 | 182 ± 5 | 55 | 18 | 22·9 ± 7·6 |
| | ,, | 3·0 | 184 ± 8 | 56 | 15 | 19·2 ± 3·9 |
| | ,, | 5·0 | 235 ± 13 | 71 | 18 | 19·3 ± 4·9 |
| Histamine-depleted | Saline | 1 to 3 | 411 ± 9 | 100 | 18 | 7·5 ± 2·5 |
| | 48/80 | 1·5 | 206 ± 11 | 50 | 18 | 5·9 ± 3·5 |
| | ,, | 3·0 | 251 ± 12 | 61 | 18 | 7·5 ± 2·7 |
| | ,, | 5·0 | 315 ± 19 | 77 | 14 | 7·0 ± 1·3 |
| Demedullated (36–56 days) | Saline | 1 to 3 | 276 ± 13 | 100 | 8 | 26·4 ± 9·3 |
| | 48/80 | 1·5 | 167 ± 8 | 60 | 9 | 19·2 ± 7·3 |
| | ,, | 3·0 | 160 ± 14 | 58 | 8 | 13·3 ± 5·6 |
| | ,, | 5·0 | 148 ± 10 | 54 | 6 | 15·8 ± 17·0 |
| Demedullated (6 months) | Saline | 1 to 3 | 369 ± 15 | 100 | 9 | 21·1 ± 5·6 |
| | 48/80 | 1·5 | 244 ± 12 | 66 | 8 | 12·3 ± 3·3 |
| | ,, | 3·0 | 221 ± 20 | 60 | 8 | 13·4 ± 3·4 |
| | ,, | 5·0 | 261 ± 24 | 71 | 9 | 15·5 ± 7·0 |
| Rats given 48/80 pretreated with adrenaline mucate (1 μg./g.). | Saline | 1 to 3 | 279 ± 16 | 100 | 6 | — |
| | 48/80 | 1·5 | 130 ± 8 | 48 | 6 | — |
| | ,, | 3·0 | 126 ± 12 | 45 | 4 | — |
| | ,, | 5·0 | 194 ± 13 | 70 | 6 | — |

tissue histamine was depleted with 48/80. The skin histamine was never reduced to undetectable amounts, but it was diminished to about 12% of normal (Table I).

The subcutaneous injection of 0.5 mg./100 g. body weight of 48/80 in these histamine-depleted animals produced none of the signs seen in normal animals. There was no further significant reduction of the already low histamine value of the skin. The pattern, but not the magnitude, of the adrenal ascorbic acid response was different. One and a half hr. after 48/80 the adrenal ascorbic acid had fallen from 411 mg./100 g. gland to 206 mg./100 g., or 50% of normal. In contrast with the effect in normal animals, the adrenal ascorbic acid level in histamine-depleted rats began to return towards the control level 3 hr. after 48/80. The average value for the adrenal ascorbic acid at this time was 251 mg./100 g. of gland, or 61% of normal. After 5 hr. the ascorbic acid level was 315 mg./100 g., or 77% of the control value (Table I).

*Effect of 48/80 in Rats with Demedullated Adrenal Glands.*—The animals were taken for experiment between 36 and 56 days after demedullation. In some rats only one gland had regenerated ; in a few, one of the glands was very large and the other very small. No glands under 5.0 mg. were used, as they seldom had ascorbic acid levels comparable with those of their larger partners.

In the demedullated animals the test dose of 48/80 produced great prostration, cyanosis, oedema, and itching. The symptoms of distress were more marked than in normal rats, being aggravated by the absence of the adrenal medullary secretion. Twenty per cent. of the animals died from 1½ to 4 hr. after the test dose. In normal rats the signs did not persist to the end of the experiment, but in demedullated animals they were still present 5 hr. after the test dose. The response of the adrenal ascorbic acid differed from that in normal rats, as the level fell continuously throughout the experiment (Table I).

The abdominal skin histamine was depleted, indicating a considerable release of histamine from the tissues. It should be noted, however, that the control level of 26.4 μg. histamine base/g. skin was lower than in normal animals and the depletion was proportionately less (Table I).

*Effect of 48/80 in Rats Pretreated with Adrenaline.*—The result of the experiment on demedullated rats suggested that the medullary secretion might possibly antagonize the effect of 48/80 on the adrenal ascorbic acid. To test this possibility, 1 μg./g. adrenaline mucate (" Hyperduric Adrenaline ") was injected subcutaneously in normal animals immediately before the usual dose of compound 48/80.

As in untreated normal rats there was prostration, cyanosis, oedema, and itching, but the signs were less severe, and they subsided 1½ hr. after the 48/80. Nevertheless the effect on the adrenal ascorbic acid was not reduced by pretreatment with adrenaline. The lowest levels were reached

1½ and 3 hr. after the test dose and were 48% and 45% of the control level respectively. After 5 hr. the level had risen to 70% of the control value (Table I). The figures indicate that pretreatment with adrenaline does not antagonize the effect of 48/80 in causing adrenal cortical activity, but rather serves to reinforce it.

*Rats with Demedullated Adrenal Glands and a Low Tissue Histamine.*—It was not possible to deplete the tissue histamine of demedullated rats by the procedure described, as they were killed by intraperitoneal injections of 48/80. However, it has been shown that with advancing age the skin histamine of the rat is reduced (Hardwick, 1954; and Nasmyth, 1954). Accordingly, a group of rats was demedullated one week after weaning and then kept for 6 months before being taken for experiment.

In these animals the 48/80 produced only mild oedema, cyanosis, and itching, and sometimes it was difficult to distinguish the effect from that of saline injections. The response of the adrenal ascorbic acid was not significantly different from that in normal animals. One and a half hours after injection of 48/80 the ascorbic acid level had fallen from 369 mg. to 244 mg./100 g. gland, representing 66% of the resting value. After 3 hr. the level was 221 mg./100 g., or 60% of the control value; but after 5 hr. it had begun to recover and stood at 261 mg./100 g., or 71% of the control value (Table I).

In previous work (Nasmyth, 1954) the abdominal skin of 7-month-old rats had been found to contain about 12 μg. of histamine base/g. skin. In the present experiments the tissue histamine of the control animals was not as low (21.1 μg./g. skin) as might have been expected; and was reduced to 12.3 μg./g. 1½ hr. after the injection of 48/80 (Table I).

*Effect of 48/80 on the Rat's Blood Pressure.*—Though the values for the abdominal skin histamine gave some indication of the activity of 48/80 in releasing histamine from the tissues, it was no guide to the physiological activity of the released histamine. In an attempt to determine this, the effect of intravenous doses of the compound on the rat's blood pressure was investigated.

In three experiments with normal rats the mean blood pressure was 129 mm. Hg. The intravenous injection of 0.5 mg./kg. produced a fall in pressure in about 8 sec. This was followed immediately by a rise in pressure and then by a precipitous and persistent fall. The lowest point was always reached about 15 min. after the injection. During the following 30 min. the pressure rose a little and then gradually fell again during 1½ hr. observation. The pressure did not rise again, but it was not possible to say whether this was caused by the drug or by deterioration of the preparation. The average maximal fall in pressure in the three experiments was 82.5 mm. Hg and was reached about 15 min. after the injection of the drug. A typical tracing is shown in Fig. 1 (A, B, and C).

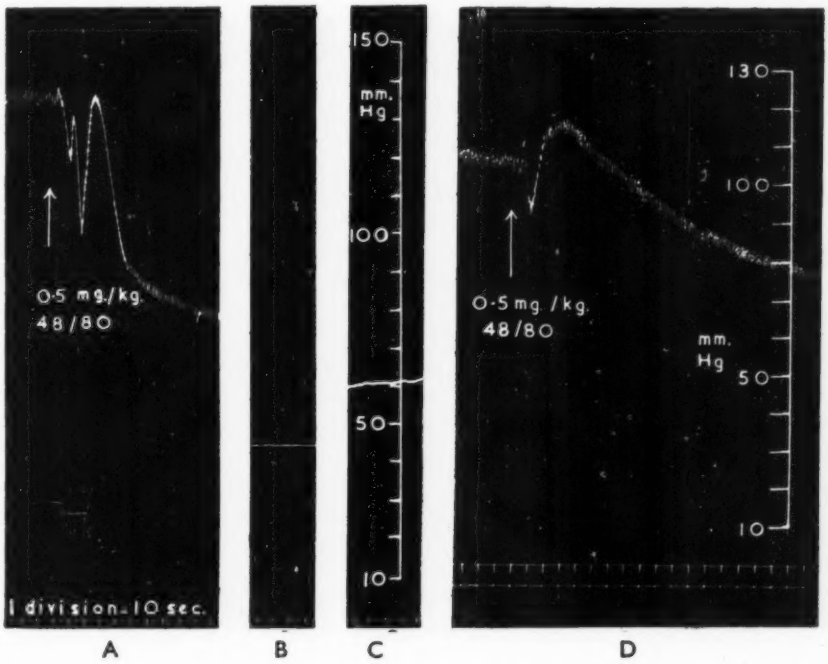


FIG. 1.—Effect of intravenous 48/80 on the arterial blood pressure of rats anaesthetized with urethane. A, B and C, normal rat; B, 15 min., and C, 47 min. after injection. D, typical response to 48/80 in histamine-depleted rat.

In 3 experiments with histamine-depleted rats the pattern of events was similar, but the fall in blood pressure was less precipitous and less extensive (Fig. 1D). The mean initial pressure was 117 mm. Hg, and the average maximal fall was 31.6 mm. Hg. One hour after the injection of 48/80 the pressure began to fall

steadily, and, again, it was not possible to say whether it was due to the drug or to deterioration of the preparation.

In three rats which had been demedullated for 6 months the average blood pressure was 102 mm. Hg and the pulse pressure was smaller than in normal and histamine-depleted rats. After an intravenous injection of 0.5 mg./kg. of 48/80 the blood-pressure response followed a similar pattern to that seen in the other two groups, but the fall was more precipitous and death invariably occurred between 20 and 30 min. after the dose. A typical record is shown in Fig. 2.

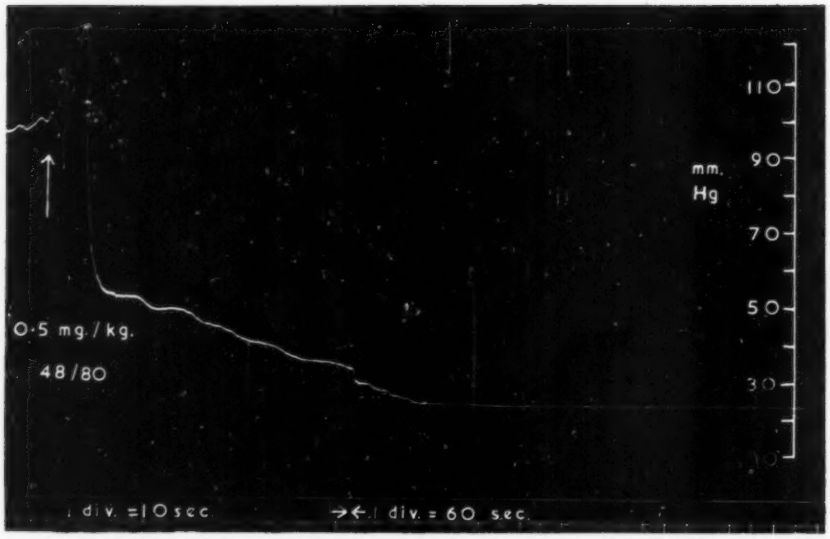


FIG. 2.—Effect of intravenous 48/80 on the arterial blood pressure of rat with adrenal glands demedullated 6 months previously. Urethane anaesthesia. The abrupt fall in pressure 25 min. after the injection (extreme right-hand end of record) indicates death.

## DISCUSSION

The present experiments show that the activity of the adrenal cortex of the rat in response to an injection of the potent histamine liberator, compound 48/80, is modified when the tissue histamine has been depleted. The modification consists of a less prolonged reduction of the adrenal ascorbic acid, which could be explained on the assumption that the "substance" producing the "stress" is reduced. This assumption is based on the observation by Hodges (1953) that in rats a subcutaneous injection of 20 μg./100 g. body weight of adrenaline produced a fall in the adrenal ascorbic acid which was as great as, but less prolonged than, that caused by 40 μg. In the present instance, the dose of 48/80 was the same in normal and histamine-depleted animals, and, since the effect of the drug on the rat's blood pressure was less in histamine-depleted rats than in normal, it seems likely that the reduced effect on the adrenal ascorbic acid was due to a reduced release of histamine into the circulation. The difference in the depressor action of 48/80 in the normal and the histamine-depleted rat is certainly due to the fact that in the depleted rat less histamine is released. It is, however, by no means certain that the reduced depressor action which remains in the depleted rat is at all caused by histamine release. However, Brocklehurst (personal communication, 1954) has shown that the local area of "blueing" produced at the site of a subcutaneous injection of 48/80 when Pontamine Sky Blue is injected intravenously is less in histamine-depleted than in normal animals, but is, nevertheless, still present. It seems likely, therefore, that at least some of the remaining effect of 48/80 on the blood pressure of the depleted rat is due to released histamine. Release of histamine, therefore, may well play some part in the effect of 48/80 on the adrenal cortex in the histamine-depleted rat, but it is not possible to say how important this release is for the reduction of ascorbic acid in these animals.

The difference between the effects of 48/80 on the ascorbic acid content of the adrenal cortex of normal and histamine-depleted rats is small, probably because histamine release is not eliminated. It is therefore unlikely that it will be possible to show whether or not released histamine plays a part in "non-specific stress" until some way of completely eliminating the release of tissue histamine is available.

The results obtained in animals with demedullated adrenal glands show that a subcutaneous dose of 0.5 mg./100 g. body weight of 48/80 is more toxic to rats with adrenal glands demedullated 35–56 days previously than it is in normal animals. On the other hand, if the animals are kept for 6 months after demedullation, the response of the adrenal cortex to 48/80 does not differ significantly from that in normal animals. Since adrenaline does not appear to antagonize the effect of 48/80, it seems likely that the more toxic effect of 48/80 in the rats demedullated 36–56 days previously is a sign of inadequate regeneration of cortical tissue, although this interval has been regarded by various authors using different forms of stress (Vogt, 1952; Hodges,

1953; Nasmyth, 1951, 1954) as being adequate. The stresses employed in these previous experiments, however, were probably less severe than the stress used in the present work, and this may account for the fact that no signs of inadequate regeneration were observed. There is also the possibility that the difference in age between the two groups of animals has affected the result.

## Summary

1. The subcutaneous injection of 0.5 mg./100 g. body weight of 48/80 causes a profound fall in the ascorbic acid content of rats' adrenals.

2. In histamine-depleted rats 48/80 causes a less prolonged fall in the adrenal ascorbic acid than in normal animals.

3. In rats with demedullated adrenals 48/80 causes a more prolonged effect on the adrenal ascorbic acid content when given 36–56 days after the operation. When given six months after the operation, the effect of 48/80 is not significantly different from that in normal animals. This suggests that a period of 36–56 days is insufficient for adequate regeneration of the cortical tissue in demedullated rats.

4. The effect of 48/80 on the blood pressure of normal, demedullated, and histamine-depleted rats is described.

5. It is concluded that released histamine plays some part in the effect of 48/80 on the adrenal cortex.

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# THE FORMALDEHYDE DERIVATIVES OF AMINO ACIDS AND THE MODE OF ACTION OF A FORMALDEHYDE-TYROSINE DERIVATIVE AGAINST INFLUENZA A VIRUS *IN OVO*

BY

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An effective chemotherapeutic agent against the smaller viruses has not yet been found. The limited activity which has been claimed for a variety of substances (reviewed by Hurst, 1953; Dickinson, 1954) is usually only shown at or near the toxic dose, or else activity is confined to tissue culture or bacteriophage tests. Lack of knowledge about protein and nucleic acid synthesis in general suggests that it will be some time before a rational approach to virus chemotherapy can be made. Some problems of protein synthesis are discussed by Chantrenne (1953) and Synge (1953).

Gale and Folkes (1953) found that chloramphenicol, chlortetracycline and oxytetracycline inhibited protein synthesis by washed suspensions of *Staphylococcus aureus*. These antibiotics are effective inhibitors of the growth of the larger viruses, and it may be, though there is no direct evidence, that they inhibit protein synthesis by the larger viruses. Chloramphenicol also inhibited the multiplication of T1 phage of *Escherichia coli* (Edlinger, 1951; Bozeman, Wisseman, Hopps, and Danauskas, 1954), but only at concentrations affecting bacterial growth. Further evidence that it may be possible to attack viral protein synthesis is suggested by the work of Ackerman (1952) and Ackerman and Maassab (1953), who found that certain $\alpha$-aminosulphonic acids affected the proliferation of influenza virus. Synthetic lysine polypeptides had slight activity against influenza A and mumps viruses (Rubini, Rasmussen and Stahmann, 1951; Green and Stahmann, 1953). Eaton, Magasanik, Perry, and Karibian (1951) found that the basic amino acids, arginine, lysine, and ornithinine had a slight action against influenza and mumps in tissue culture.

As part of a programme of investigations into the chemotherapy of virus diseases, amino acids and various derivatives (particularly formaldehyde derivatives) have been investigated, using influenza A and a bacteriophage of *Pseudomonas pyocyanea* as test viruses. One of the disadvantages of studying formaldehyde (F) derivatives is that many are very unstable; however, derivatives prepared at 70°, although more complex since more bonds react, are usually more stable. If dissociation occurred the free F formed would be markedly toxic and mask any other activity. However, traces of free F are readily detected on bacteriophage assay plates as zones of host (*Ps. pyocyanea*) inhibition; in such experiments anti-phage activity cannot be assayed.

A formaldehyde-tyrosine preparation (TF) has been found to be active against influenza virus in eggs but not in mice. The mode of action of this substance in eggs is described.

## METHODS

*Formaldehyde Derivatives.*—The methods of Fraenkel-Conrat *et al.* (1945, 1946, and 1948) were used. One g. amino acid in 10 ml. buffered F solution (containing 10 ml. 40% formaldehyde soln. + 60 ml. 0.1 M phosphate-citrate buffer *p*H 8.0) was put in a tightly capped 1-oz. bottle for four days at 70°, with occasional shaking. After cooling, 5–6 vol. ice-cold acetone were added and the precipitate removed, redissolved, and reprecipitated by acetone once or twice (depending on the yield). Supernatant fluids were not investigated. Yields varied, but there was usually sufficient material for testing (Table I): glycine, leucine, and tyrosine gave good yields. The derivatives were usually colourless or yellow oils, but crystalline solids (e.g., valine and $\beta$-phenylalanine derivatives) were sometimes obtained. The method for the tyrosine derivative is given in detail later: this was the only compound showing activity. A cysteine derivative, thiazolidine-4-carboxylic acid, was prepared by the method of Ratner and Clarke (1937).

*Copper Derivatives.*—Amino acids and hydrolysates were boiled with excess basic copper carbonate until there was no further effervescence. After filtration the soluble complexes were evaporated and where possible crystallized from hot water. Insoluble derivatives were not investigated.

TABLE I

ANTI-VIRAL ACTION OF AMINO ACIDS AND THEIR FORMALDEHYDE DERIVATIVES

| Amino Acid, etc. | Activity | | Formaldehyde Derivatives | | |
|---|---|---|---|---|---|
| | | | Yield | Activity | |
| | Influenza | Phage | (+ to +++) | Influenza | Phage |
| Glycine .. | 0 | 0 | +++ | 0 | 0 |
| DL-Alanine .. | 0 | 0 | + | 0 | 0 |
| DL-Leucine .. | 0 | 0 | +++ | 0 | 0 |
| DL-Norleucine | 0 | 0 | – | — | — |
| DL-Isoleucine .. | 0 | 0 | + | 0 | 0 |
| DL-Lysine .. | 0 | 0 | + | 0 | 0 |
| L-Arginine .. | 0 | 0 | ++ | 0 | 0 |
| DL-Valine .. | 0 | 0 | + | 0 | 0 |
| DL-Serine .. | 0 | 0 | ++ | 0 | 0 |
| DL-Proline .. | 0 | 0 | ++ | 0 | 0 |
| DL-Glutamic acid | 0 | 0 | + | 0 | 0 |
| DL-Aspartic ,, | 0 | 0 | + | 0 | 0 |
| DL-Asparagine | 0 | 0 | + | 0 | 0 |
| DL-Tryptophane | 0 | 0 | + | 0 | 0 |
| L-Tyrosine .. | 0 | 0 | +++ | A(4–16) | 0 |
| L-Histidine .. | 0 | 0 | + | 0 | 0 |
| DL-Methionine | 0 | 0 | + | 0 | 0 |
| L-Cystine .. | 0 | 0 | + | 0 | 0 |
| L-Cysteine .. | 0 | 0 | + | 0 | 0 |
| Glycylglycine | 0 | 0 | ++ | 0 | 0 |
| Polylysine (mol. wt. 1600) .. | 0 | A(5) | + | 0 | 0 |
| Polylysine (mol. wt. >16,500) | 0 | A(5) | + | 0 | 0 |
| DL-β-Phenylalanine .. | 0 | 0 | ++ | 0 | 0 |
| Nitroarginine | 0 | 0 | + | 0 | 0 |
| Glycocyamine | 0 | 0 | ++ | 0 | 0 |
| Betaine .. | 0 | 0 | – | | |
| Taurine .. | 0 | 0 | + | 0 | 0 |
| Sarcosine .. | 0 | 0 | + | 0 | 0 |
| DL-Benzoylalanine .. | 0 | 0 | + | 0 | 0 |
| Acetamide .. | 0 | 0 | +++ | 0 | 0 |

A = active. 0 = inactive at ½ the dose toxic to the host, or at 10 mg./egg. 0 = activity factor expressing the chemotherapeutic ratio. — = no derivative obtained.

*Other Derivatives.*—The following were prepared: nitroarginine (Kossel and Kennaway, 1911); acetamidomethylene compound of alanine (Fraenkel-Conrat, 1948) and that of leucine by a similar method at *p*H 3.0; hydantoins and *N*-carbamyl derivatives of tyrosine and leucine (Dakin, 1910) and of arginine (Boon and Robson, 1935).

*Tests against Influenza A Virus.*—0.2 ml. of a series of 4-fold dilutions of a 10% soln., or suspension, of the drug in water were injected into the allantoic sac, unless otherwise stated, of 10–12 day embryonated eggs, followed 1 hr. later by 10–100 egg-infecting doses (EID) of influenza A virus (PR8 strain). After a further 48 hr. incubation the allantoic fluids were tested for their ability to haemagglutinate fowl red blood cells, by the method of Salk (1944). Control eggs had titres (expressed as reciprocals) of 1024 or 2048. Treated eggs with titres less than 10 were titrated for infectivity. All infectivity titres refer to 0.25 ml. volumes of inocula. Tests for possible inhibition of haemagglutination by the drug were made by adding 4 u. haemagglutinin to 0.25 ml. allantoic fluid from treated eggs, plus 0.25 ml. of 0.5% fowl cells. Tests for false haemagglutination by the compounds were made by adding 0.25 ml. fluid from treated eggs to 0.25 ml. blood.

In the tests, groups of three or four eggs were used and activity was only considered significant if it occurred in all eggs of a group at a dose which did not kill the embryo. Many compounds, including F itself, showed sporadic activity at or just below the toxic dose in these, and other, investigations, but the study of such border-line activity needs large numbers of eggs.

*Tests against Bacteriophage.*—A virulent phage, Pc, of *Pseudomonas pyocyanea* was used as the test virus. The lactate medium and method of Chantrill, Coulthard, Dickinson, Inkley, Morris, and Pyle (1952) were used for quantitative tests, the results of which were expressed as $\frac{\text{mg./ml. inhibiting phage}}{\text{mg./ml. inhibiting host}}$, abbreviated to a factor. For preliminary testing, to rule out inactive compounds, and to note host inactivators (usually due to F liberation), cup plate tests, using lactate medium with 0.5% leucine and 1.5% agar, were employed; the phage and host content were arranged to give almost confluent lysis. After diffusion overnight at 5° plates were incubated for 18 hr. at 37°. Zones of phage inhibition appeared as zones of host growth.

## RESULTS

Results appear in Table I and indicate that significant anti-viral activity was shown only by the formaldehyde-tyrosine preparation. Several other formaldehyde derivatives showed anti-influenza activity at the dose toxic to some eggs, but these were not studied.

The copper derivatives of glycine, DL-glutamic acid, DL-alanine, DL-aspartic acid, L-arginine, DL-valine and glycylglycine were all inactive. The *N*-carbamyl derivatives and hydantoins of DL-leucine, L-tyrosine, and L-arginine, the *N*-(*N*-acetamidomethylene) derivatives of alanine and leucine, and thiazolidine carboxylic acid were also inactive.

Polylysine was included in these tests because it had been reported to inhibit influenza A and mumps viruses *in ovo* (Rubini, Rasmussen and Stahmann, 1951; Green and Stahmann, 1953). Both samples tested (mol. wt. 1600 and >16,500) were inactive. When the allantoic fluids of treated eggs were titrated by the haemagglutination technique, no difference in the titres was found. Both samples agglutinated fowl cells, when diluted in saline (down to concentrations of 1 μg./ml.), but

the allantoic fluid of eggs receiving even 3.2 mg. did not cause agglutination. Since the maximum non-toxic dose to eggs was 0.8 mg., the tests were not invalidated. The slight anti-phage action was not impressive in view of the marked anti-bacterial action.

*The Action of Formaldehyde-Tyrosine on Influenza A Virus*

TF was the only substance found significantly active against influenza A virus *in ovo* ; the chemotherapeutic ratio was 4–16. The action of TF was almost certainly not due to formaldehyde (F) liberation, for the following reasons:

1. A solution of F was only active at a dose toxic to some of the eggs or at half this dose.

2. The activity of TF solutions was unaffected by boiling, whereas one would expect dissociation to increase.

3. TF was inactive against bacteria in cup-plate tests, whereas traces of F could be detected.

*Preparation and Properties.*—The preparation required certain experimental conditions. Tyrosine had to be in excess ; reducing its proportion to 1/10th resulted in a yield of yellow crystals, which were inactive. When the reaction temperature was reduced from 70° to 37°, inactive pink crystals were produced. Probably the initial reaction was followed by further coupling with excess tyrosine. Eventually the following process was standardized.

Batches of L-tyrosine (2 g.) were suspended in buffered F (20 ml.) as in the standard process (see Methods). During incubation at 70° a considerable amount of free tyrosine disappeared and the liquor turned first pink and then bright yellow. After cooling and filtering off the unchanged tyrosine the filtrate was cooled to +5° and then added, with vigorous agitation, to six times its volume of cold acetone. The yellow precipitate was centrifuged down, redissolved in the minimum amount of water and again precipitated by cold acetone ; some batches were precipitated three times with acetone. The final precipitate was suspended in dry acetone, filtered off and washed with acetone and ether. For preparing 25-g. batches of TF, 25 separate 2-g. amounts of tyrosine were used, as the yields were poor if larger quantities were handled. Yields of the reprecipitated material were usually about 60% of the weight of the original tyrosine.

The active substance was a yellow amorphous powder which melted gradually, just above room temperature, to a brown liquid. It became resinous on standing for some weeks at room temperature, but this gummy material was still active. It was very soluble in water, a 25% solution being readily prepared, but insoluble in common organic solvents.

*Action on Influenza A Virus* in ovo

Nine batches of TF, prepared on different occasions, all showed activity at 10 mg./egg ; up to 80 mg. of some batches could be given via the allantoic sac or yolk sac without causing death of the embryo. One batch was active at all doses from 5–80 mg./egg. Its action was not affected by serum broth and it was active when given 1, 2, and 4 hr. after, or 24–48 hr. before, infection. Eighty mg./egg via the yolk sac was inactive against virus inoculated via the allantoic sac or chorioallantoic membrane.

Twenty mg. TF/egg was active against 100 or 10,000 EID virus both in the 48 hr. allantoic sac test and also when the test was prolonged to 96 hr. Against either dose of virus, the haemagglutinin titres of allantoic fluid from treated eggs were always $<2$, whereas controls were 1024–2048. For the 100 EID inoculum, the infectivity titres were: treated $10^0$–$10^2$, controls $10^6$–$10^8$. A second dose of virus, given to eggs that had been treated (and infected) for 48 hr., did not grow. The washed cells of the chorioallantoic membrane of treated eggs were still capable of supporting the growth of virus in tissue culture (Mrs. M. J. Thompson, personal communication). The treated allantoic fluid did not inhibit haemagglutination.

*Action on Extracellular Virus* in ovo (*Contact Test*)

1. Five eggs, infected 48 hr. previously and incubated, were treated with 20 mg. TF/egg and incubated for 24 hr. to find the effect on free virus. The titre of the allantoic fluid fell from $10^6$–$10^7$ to $10^4$ ; haemagglutination titres fell from 1024–2048 to $<2$, $<2$, 2, 2, 4. Control eggs showed no fall in haemagglutination titre and were still infective at $10^{-6}$.

2. Eggs which had been given 20 mg. TF/egg 1 hr. previously were infected with 10,000 EID virus and re-incubated for 1 hr.; the allantoic fluids were then removed and 0.25 ml. samples injected into groups of 3 eggs. No virus was detected in 2 treated eggs, but it was present in the 3rd egg. The 3 control eggs were infective at 10° but not at $10^{-2}$ (except for 1/3 eggs). This drop was expected because of adsorption on to the cells, the tests being designed to indicate effects on adsorption. Using 10–100 EID, no virus was detected in either control or treated eggs at 1 hr.

*Action on Extracellular Virus* in vitro (*Contact Test*)

Neither 50 mg. TF/ml. nor 10 mg./ml. inactivated 1,000 EID virus within 15 min. at 37°

in M/15 phosphate-citrate buffer, *p*H 7.2. However, when the contact period was 1 hr. the titre fell to 10 EID. These experiments were typical of many *in vitro* contact tests, giving different results according to the conditions.

*Chemotherapeutic Tests in Mice*

TF was not toxic to mice when given subcutaneously in a single dose of 5 mg./g. Small-scale *in vivo* tests against influenza A were done, giving the drug subcutaneously, intraperitoneally, intranasally, and as an aerosol, in varying doses.

Mice, in groups of 10 or 20, were infected with mouse-adapted influenza A virus (PR8) intranasally under light anaesthesia. Treatment was given twice daily, starting 3 hr. before infection, and continued for 7–10 days, when survivors were killed and the lungs assessed for consolidation (values 0–4, death with lungs totally consolidated, 5). A daily dose of 4 mg./g. was toxic parenterally, but 2 mg./g. was well tolerated. The drug was very toxic when given as a 2% solution intranasally. Given as an aerosol (1% solution for 1 hr. twice daily), it was better tolerated but had no protective value. A preliminary test, giving the drug subcutaneously in 2 daily doses of 1 mg./g. each, had suggested a reduction of lung lesions (controls, 6/10 survivors, average lung assessment 3.6; treated, 8/10 survivors, average lung assessment 1.7; $P < 0.01$ for difference in lung-lesion assessment). These results were not confirmed when the test was repeated (controls, 8/20 survivors, average lung assessment 4.2; treated, 4/21 survivors, average lung assessment 4.7).

## Discussion

The commonly used allantoic sac test of anti-viral activity is useful if regarded as a primary screening test. The few compounds showing activity can then be investigated further; the results usually eliminate the need for large-scale animal tests, especially if the mouse toxicity figures indicate that the concentrations active in eggs are not likely to be attained in animals.

Ignoring the host defence system, which is not well developed in the egg, the possible modes of action of an anti-viral compound are either by the direct inactivation of extracellular virus (contact action), or by the prevention of adsorption, multiplication or release of virus. If the drug is given by the same route as the virus, even several hours after infection, a contact action cannot be excluded. The usual arbitrary *in vitro* contact tests, varying in time and medium, may be very misleading, particularly as these tests have usually to be made on a high concentration of virus (to allow dilution of the drug before injection into the egg). In chemotherapeutic tests only 10–100 EID are given, and even a slight contact action may reduce the virus concentration to a sub-infective level. A compound exerting its effect by contact action on free virus can be detected by the fact that no free virus is present when the tests are read at 48 hr. The only alternative explanation for complete absence of free virus assumes that the unadsorbed fraction of the inoculum contains too little virus to be detected, and also that viral multiplication (or release) is completely suppressed.

An effect on adsorption can be detected by finding the level of virus 1–4 hr. after the injection of an inoculum sufficiently heavy to leave a measurable residual level after adsorption. If no adsorption occurs the initial inoculum remains free and is higher than in control eggs. Henle (1953) stated that adsorption was practically complete in 1 hr., but Horsfall (1954), whose results had not been published when the TF work was done, found the minimum free virus level at 4 hr. If the virus titre is lower than in control eggs a contact action is again indicated.

If the virus titre at 48 hr. is equal to or above the residual virus at 1 hr. but significantly less than in control eggs, then an inhibition of viral multiplication (or release) is indicated—assuming that the cells of the allantoic sac are still capable of supporting viral growth (tested in tissue culture). The titre may rise on further incubation, indicating an effect on the *rate* of multiplication, or possibly the gradual inactivation of the drug; this effect, however, may be associated with a drug concentration which is toxic to the host and is then of little interest. On all types of action there may be a superimposed contact action and this is illustrated by the results for TF, which was one of the very few compounds active in the primary test. It had a chemotherapeutic ratio in eggs of 4–16 and was active in the presence of serum broth. It was stable and easy to prepare and therefore suitable for further investigation.

TF-treated eggs contained small amounts of free virus at 48 hr., the amount being greater than at 1 hr. after infection; for 10–100 EID inocula no virus was found at 1 hr., but the titre was $10^0$–$10^2$ at 48 hr., compared to $10^6$–$10^7$ for control eggs, and it did not rise on further incubation. Contact action was indicated by: (1) the results at 1 hr., after 10,000 EID virus (titre less than in control eggs); (2) the *in ovo* contact test. It is not possible to discriminate between an effect on adsorption and

one on multiplication, in view of the contact action. However, the ratio of titres of control fluids to TF treated fluids in the *in ovo* contact test was $10^6$–$10^7/10^4$ by infectivity (1024–2048/2 by haemagglutination), while the corresponding ratio in the chemotherapeutic tests was $10^6$–$10^7/10^0$–$10^2$. The difference in the ratios, together with the slight increase in virus between 1 and 48 hr., suggests that only part of the action of TF was due to inactivation of free virus.

TF was inactive when given by the yolk sac. It may be a large molecule, since it appears to be formed by repeated reactions of excess tyrosine with intermediate products; permeability effects may account for its inactivity when given by this route. The effective concentration, calculated from egg studies, could only just be (theoretically) attained in mice at the maximum non-toxic dose; for this reason only small-scale animal tests were done.

## Summary

1. Amino acids and derivatives (mainly formaldehyde derivatives) were tested against influenza A virus in eggs and against a bacteriophage of *Ps. pyocyanea*.

2. A formaldehyde-tyrosine derivative was active against influenza *in ovo*; the chemotherapeutic ratio was 4–16. It was not active in mice.

3. Studies on the mode of action of this substance suggested that only part of the effect was due to inactivation of extracellular virus.

4. By considering the results of various *in ovo* experiments it is often possible to decide whether a substance found active *in ovo* is worth testing in animals.

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# THE ACTION OF LYSERGIC ACID DIETHYLAMIDE ON MAMMALIAN CHOLINESTERASES

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The psychological effects produced by administration of lysergic acid diethylamide (LSD) to man are now well known (see Stoll and Hofmann, 1943; Stoll, 1947, 1949; Condrau, 1949). Considerable information is also available about the general pharmacological effects in animals, but there is very little evidence bearing on the possible biochemical mode of action of this compound. Mayer-Gross, McAdam and Walker (1951, 1952) have described a slight transitory increase in the glucose and hexosemonophosphate levels in the blood following the administration of LSD, but carbohydrate metabolism did not otherwise appear to be affected; they suggested that the psychological symptoms may in part be due to an interruption of carbohydrate breakdown resulting from a block at the hexosemonophosphate stage. Liddell and Weil-Malherbe (1953), however, concluded that the effects of LSD on the blood sugar level were hardly significant, although they did describe changes in the blood adrenaline level.

Because of a similarity in molecular structure between lysergic acid and tryptamine, and because 5-hydroxytryptamine is known to be present in certain parts of the brain (Amin, Crawford and Gaddum, 1952), Gaddum (1953) carried out experiments to determine whether any interaction existed between these two drugs; he found that small amounts of LSD inhibit the action of 5-hydroxytryptamine on the rat's uterus.

Lewis and McIlwain (1954) have studied the action of LSD on the respiration and glycolysis of guinea-pig cerebral cortex; they found that when the tissue was stimulated by electrical pulses both these processes were inhibited by concentrations between $3 \times 10^{-6}$M and $10^{-4}$M. Similar concentrations of both ergotoxine and dihydroergotamine were, however, also found to produce a similar degree of inhibition. They also reported that this effect of LSD was not prevented by the simultaneous presence of 5-hydroxytryptamine.

A further approach to the mode of action of this compound was initiated by Poloni and Maffezzoni (1952); these workers studied the levels of acetylcholine in the brains of guinea-pigs treated with bulbocapnine, mescaline and LSD; they found that LSD caused an increase in the acetylcholine level of the brain. Animals treated with bulbocapnine showed decreased amounts, whereas with mescaline the level was unchanged.

In view of this last report we have studied the action of LSD on some esterases, including the cholinesterases, of man and laboratory animals. Since LSD is a synthetic derivative of one of the constituents of ergot, a few experiments have also been carried out with ergotoxine, ergotamine, dihydroergotamine and ergometrine.

A preliminary report on this work was given to the Biochemical Society on July 16, 1954 (Thompson, Tickner and Webster, 1954).

## METHODS

*Estimation of Esterase Activity*

Esterase activity was determined manometrically at 38° C. and at *p*H 7.4 using the Warburg technique (Ammon, 1933).

*Substrates*

(1) Acetylcholine perchlorate (ACh) (British Drug Houses, Ltd.).

(2) Butyrylcholine perchlorate (BuCh), prepared in this laboratory.

(3) Acetyl-β-methylcholine chloride (MCh) (Savory & Moore, Ltd.).

(4) Tributyrin (TB) (British Drug Houses, Ltd.).

The choline esters were dissolved in 0.025 M-$NaHCO_3$ solution immediately before use. The tributyrin was pipetted directly into the side-arm of the Warburg flasks (0.03 ml./flask), and was covered with 0.17 ml. of 0.025 M-$NaHCO_3$.

*Materials*

(1) Human serum.

(2) Human erythrocytes, washed once with saline and resuspended in distilled water, the final volume equalling the original volume of blood.

(3) Various areas were dissected from human brains obtained from cadavers. Because of the relatively high level of pseudo-cholinesterase activity in the white fibre tracts of human brain (Ord and Thompson, 1952, and later unpublished observations), the cerebral white matter (frontal lobe), internal capsule and corpus callosum were used as a source of this enzyme. The caudate nucleus was used for estimation of true cholinesterase.

(4) For experiments with rat, guinea-pig, rabbit, and chicken brain, the whole brain with the exception of the cerebellum was used. With monkey brain (*Macacus rhesus*) the internal capsule, corpus callosum and the white matter from the cerebrum were dissected and either pooled or the internal capsule used separately for pseudo-cholinesterase determinations. Cerebral cortex was used as a source of true cholinesterase.

In each experiment a weighed amount of the brain tissue was homogenized in 0.025 M-$NaHCO_3$. The homogenates, whose concentrations were chosen to give suitable $CO_2$ evolutions when measured over 0–60 min., were added to the main bulbs of the Warburg flasks.

*Ergot Derivatives*

(1) Lysergic acid diethylamide (Sandoz Products, Ltd.).

(2) Ergotoxine ethanesulphonate (British Drug Houses, Ltd.).

(3) Ergotamine tartrate (Sandoz Products, Ltd.).

(4) Dihydroergotamine methanesulphonate (Sandoz Products, Ltd.).

(5) Ergometrine maleate (Burroughs, Wellcome & Co.).

(6) Methylergometrine tartrate (Sandoz Products, Ltd.).

## Results

### *Effect of LSD on Esterases in Human Blood*

The effect of LSD on the activity of pseudo-cholinesterase in human serum was first studied, using 0.015 M-ACh perchlorate as substrate. Under these conditions $10^{-6}$M-LSD (=1 μg./ml.) caused about 50% inhibition of activity (Table I), whereas $2 \times 10^{-5}$M produced almost complete inhibition. Using 0.015 M-BuCh perchlorate as a selective substrate for pseudo-cholinesterase (Nachmansohn and Rothenberg, 1945), inhibition was again observed, although to a lesser extent than with ACh as substrate. On the other hand, the true cholinesterase in human erythrocytes was not affected by $2 \times 10^{-5}$M-LSD, and a concentration of $10^{-4}$M produced only 19% inhibition.

The early observations of Vahlquist (1935), Easson and Stedman (1937) and Richter and Croft (1942) had suggested that the hydrolysis of aliphatic esters such as tributyrin by human plasma was brought about very largely by the plasma cholinesterase. The more recent work of Adams and Whittaker (1949) disclosed the presence in human plasma of a small amount of a second enzyme capable of hydrolysing TB and triolein, but these workers also concluded that in plasma the pseudo-cholinesterase is mainly responsible for TB hydrolysis. We therefore also used TB as substrate, the hydrolysis of which was found to be inhibited by low concentrations of LSD (Table I).

TABLE I

PERCENTAGE INHIBITION OF HUMAN ESTERASES BY LSD (1 μG. LSD/3 ML. = APPROX. $10^{-6}$M)

| Enzyme Source | Substrate | LSD Concn. (μg./3 ml.), and % Inhibition Produced | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 5 | 10 | 20 | 50 | 100 |
| Plasma | ACh (0·015M) | 49 | 66 | 87 | 89 | 98 | — | — |
| ,, | ACh (0·03M) | — | — | 73 | — | — | — | — |
| ,, | ACh (0·06M) | — | — | 58 | — | — | — | — |
| ,, | BuCh (0·015M) | 24 | 40 | 63 | 74 | 84 | — | — |
| ,, | Tributyrin | 70 | 79 | 89 | 91 | 94 | — | — |
| Erythrocytes | ACh (0·015M) | — | — | 3 | 0 | 0 | 16 | 19 |

TABLE II

PERCENTAGE INHIBITION FROM $5 \times 10^{-6}$M-LSD (=5 μG. 3 ML.) WITH VARYING SUBSTRATE CONCENTRATIONS

| Enzyme Source | Substrate | Substrate Concn., and % Inhibition Produced | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0·0019M | 0·0037M | 0·0075M | 0·015M | 0·03M | 0·06M |
| Human serum | ACh | 94 | 90 | 91 | 87 | 73 | 58 |
| ,, ,, | BuCh | 88 | 90 | 71 | 63 | 36 | 19 |
| ,, erythrocytes .. | ACh | 0 | 5 | 8 | 3 | 0 | — |

Table II shows the effect of variations in the concentration of ACh and BuCh on the inhibition produced by $5 \times 10^{-6}$M-LSD. With either substrate the degree of inhibition of the pseudo-cholinesterase diminishes as the substrate concentration increases; analysis of the data according to Lineweaver and Burk (1934) indicates that LSD inhibits competitively. The true cholinesterase in the red cells, on the other hand, remained insensitive to this concentration of LSD over the range of substrate concentrations studied.

Four experiments were done to discover whether the inhibition of cholinesterase by LSD is readily reversible or not. One volume of human serum was added (*a*) to 3 vols. of 0.025 M-$NaHCO_3$, and (*b*) to 3 vols. of 0.025 M-$NaHCO_3$ containing LSD. Samples of each were taken for estimation of the inhibition produced by the LSD (final concentration = 5 μg. LSD/3 ml.). After 15 min. at room temperature samples of (*a*) and (*b*) were diluted

5, 10 and 20-fold with 0.025 M-$NaHCO_3$, and the inhibition at each dilution estimated: in the undiluted flasks cholinesterase activity was 85% inhibited; after 5, 10 and 20-fold dilution the inhibition was 46, 24 and 18% respectively.

*Effect of LSD on Brain Esterases*

The pseudo-cholinesterase in human brain was next studied, using various white fibre tract areas as well as the cerebellum as a source of enzyme and both ACh and BuCh as substrates. It will be seen from Table III that this enzyme in brain is also highly sensitive to inhibition by LSD. The true cholinesterase of human brain, on the other hand, using both caudate nucleus and the cerebellum as enzyme sources, appears to be almost completely insensitive to the concentrations of LSD listed in Table III, with either ACh or the selective substrate MCh (Mendel, Mundell, and Rudney, 1943) as substrate.

TABLE III

PERCENTAGE INHIBITION BY LSD OF HUMAN BRAIN ESTERASES

| Enzyme and Source | Substrate | LSD Concn. (μg./3 ml.) and % Inhibition Produced | | | |
|---|---|---|---|---|---|
| | | 5 | 10 | 20 | 50 |
| *Pseudo-cholinesterase* | | | | | |
| Frontal cerebrum (white) | ACh | 66 | 60 | 63 | 60 |
| " " " .. | BuCh | 54 | 69 | 83 | 88 |
| Cerebellum .. .. | BuCh | — | 44 | — | — |
| Internal capsule .. .. | BuCh | — | 76 | — | — |
| Corpus callosum .. .. | BuCh | — | 62 | — | — |
| *True cholinesterase* | | | | | |
| Caudate nucleus .. | ACh | 2 | 5 | 4 | 10 |
| " " .. .. | MCh | 2 | 2 | 6 | 8 |
| Cerebellum " .. .. | MCh | — | 0 | — | — |
| *Tributyrinase* | | | | | |
| Frontal cerebrum (white) | TB | 1 | 8 | 10 | 7 |
| Caudate nucleus .. .. | TB | 0 | 4 | 13 | 21 |

In contrast to the results obtained with human serum, TB hydrolysis by human brain appears to be very largely unaffected by these concentrations of LSD. This result agrees with earlier observations (Ord and Thompson, 1952) indicating that brain pseudo-cholinesterase does not contribute to any significant degree to the hydrolysis of TB.

TABLE IV

PERCENTAGE INHIBITION BY LSD OF BRAIN CHOLINESTERASE OF VARIOUS SPECIES

| Species | Source of Enzyme | Substrate | LSD Concn. (μg./3 ml.) and % Inhibition Produced | | | |
|---|---|---|---|---|---|---|
| | | | 5 | 10 | 20 | 50 |
| Rat .. | Whole brain minus cerebellum | BuCh | 9 | 3 | 5 | 6 |
| | | MCh | — | 0 | — | — |
| Guinea-pig | " " | BuCh | 2 | 4 | 13 | 3 |
| | | MCh | — | 0 | — | — |
| Rabbit | " " | BuCh | 12 | 11 | 23 | 37 |
| Chicken | " " | BuCh | 3 | 4 | 7 | 0 |
| Monkey | Internal capsule | ACh | 6 | — | — | — |
| | " " | BuCh | 9 | 20 | 33 | — |
| | Pooled white matter | BuCh | 8 | 29 | 35 | 61 |
| | Cerebrum (grey) | MCh | 5 | 0 | 2 | 8 |

The effect of LSD on the cholinesterases in the brains of a number of animal species has also been studied. It will be seen (Table IV) that BuCh hydrolysis by rat, guinea-pig and chicken brain is hardly affected by concentrations of LSD which produce a very substantial inhibition in human brain. The pseudo-cholinesterase of rabbit brain seems slightly more sensitive, and that of monkey brain still more so, although their individual sensitivities are considerably less than that of human brain. True cholinesterase in rat, guinea-pig, and monkey brain appears to be unaffected by the concentrations used.

The results obtained with the other ergot alkaloids studied are shown in Table V. It will be seen that ergotoxine, ergotamine, and dihydroergotamine produce only slight inhibition (0–17%) of human serum cholinesterase at a concentration of 100 μg./3 ml. Ergometrine maleate, on the other hand, is a more potent inhibitor, though considerably less active than LSD; thus 5 μg. ergometrine maleate/3 ml. causes only 16% inhibition of serum cholinesterase, as compared with 87% produced by 5 μg. LSD/3 ml. Methylergometrine tartrate, although more active than ergometrine in its action on the pregnant rabbit's uterus, causes less inhibition of pseudo-cholinesterase.

TABLE V

PERCENTAGE INHIBITION OF HUMAN SERUM CHOLINESTERASE BY ERGOT DERIVATIVES, WITH ACh AS SUBSTRATE

| Derivative | Concn. (μg./3 ml. of Derivative), and % Inhibition Produced | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 5 | 10 | 20 | 50 | 100 | 200 |
| Ergotoxine ethanesulphonate | — | — | — | — | — | 5 | 13 |
| Ergotamine tartrate .. | — | — | — | — | — | 17 | 22 |
| Dihydroergotamine methanesulphonate | — | — | — | — | — | 0 | 12 |
| Ergometrine maleate .. .. | 6 | 16 | 32 | 40 | 63 | 74 | 85 |
| Methylergometrine tartrate | 0 | 0 | 1 | 6 | 10 | 29 | 50 |

In view of Gaddum's findings (1953) quoted earlier, we have done two experiments to determine whether the simultaneous presence of 5-hydroxytryptamine affects the inhibition of pseudo-cholinesterase by LSD; $10^{-4}$M-5-hydroxytryptamine creatinine sulphate (kindly provided by Dr. E. Lester Smith) did not influence the inhibition caused by $2 \times 10^{-6}$M-LSD.

## Discussion

The foregoing results indicate that LSD is a relatively powerful inhibitor of the pseudo-cholinesterase of human plasma, approximately 50% inhibition of enzymic activity being brought about by a concentration of $10^{-6}$M. True cholinesterase in human erythrocytes is only slightly affected by concentrations that cause almost complete inhibition of pseudo-cholinesterase. The same relationship appears to hold for the cholinesterases in human brain, pseudo-cholinesterase being inhibited by concentrations of LSD which leave the true cholinesterase hardly affected. Table III shows that, when BuCh is used as substrate, increasing concentrations of LSD (up to 50 $\mu$g./3 ml.) cause increasing degrees of inhibition of brain pseudo-cholinesterase (up to 88%), whereas with ACh as substrate the inhibition remains at about 60% even though the concentration of LSD is increased tenfold up to levels which cause virtually complete inhibition of the plasma enzyme ; this is probably because with ACh as substrate both types of cholinesterase are estimated, so that even in the presence of amounts of LSD which cause complete inhibition of pseudo-cholinesterase there will still be a measurable activity owing to the LSD-insensitive true cholinesterase.

Tables I and III show that, whereas TB hydrolysis by human plasma is inhibited by low concentrations of LSD, the tributyrinase activity of brain is insensitive. As mentioned earlier, therefore, it would seem that the pseudo-cholinesterase of brain, unlike the plasma enzyme, does not hydrolyse TB.

The action of LSD differs in several respects from that of many of the more extensively studied inhibitors of cholinesterases ; thus eserine inhibits both true and pseudo-cholinesterases, whereas di*iso*propyl phosphorofluoridate (DFP), although showing some selective action towards pseudo-cholinesterase, will also inhibit true cholinesterase at slightly higher concentrations ; tri*ortho*cresyl phosphate inhibits *in vivo* both pseudo-cholinesterase and tributyrinase, although it is inactive against true cholinesterase. LSD appears to inhibit more specifically the pseudo-cholinesterase, leaving both true cholinesterase and tributyrinase in brain relatively unaffected. The seemingly rapid reversibility of the inhibitory action of LSD is another point of differentiation between this compound and the organo-phosphorus anti-cholinesterases.

The high sensitivity of human pseudo-cholinesterases to low concentrations of LSD, as compared with the pseudo-cholinesterases of the rat, guinea-pig, rabbit, chicken and even the monkey (Table IV) is of interest in connexion with the suggestions in the pharmacological and clinical literature that man is more sensitive to this compound than are most of the lower animals so far studied.

In general, our results with the other ergot alkaloids acting on human serum fall into line with those of earlier workers. Thus, Gautrelet and Scheiner (1939) claimed that ergotamine tartrate has only about 1/200th the anti-cholinesterase activity of eserine towards horse serum cholinesterase. Brügger (1938), using defibrinated cat's blood as a source of cholinesterase, concluded that both ergotamine and ergometrine were only weak inhibitors of cholinesterase. Navratil (1937), on the other hand, tested ergometrine on human serum, and obtained a degree of inhibition agreeing well with that found by us.

We cannot reach any conclusions from the work described here as to whether this action on pseudo-cholinesterase is relevant to the pharmacological effects produced by ingestion of this drug by man, although the finding of increased levels of ACh in the brains of guinea-pigs treated with LSD (Poloni and Maffezzoni, 1952) would be consistent with the presence of cholinesterase inhibition *in vivo*.

Mental changes following the administration of another cholinesterase inhibitor, di*iso*propyl phosphorofluoridate, have also been described, although it does not necessarily follow that the changes were directly due to the anti-cholinesterase action of the DFP in the brain. For example, Rowntree, Nevin, and Wilson (1950) reported that the daily administration of DFP to normal subjects and to depressive patients caused depressant effects, whereas, when administered to a number of schizophrenic patients, an activation of the psychosis was observed. Aprison, Nathan, and Himwich (1954) have also described abnormal behaviour patterns in rabbits, involving circling and compulsive turning movements, associated with inhibition of brain cholinesterase activity by injection of DFP into the common carotid artery. It must be pointed out, however, that recent evidence strongly suggests that pseudo-cholinesterase in the nervous system is a component not of the neurones but of the glial and Schwann cells (Cavanagh, Thompson, and Webster, 1954). Indeed, since the physiological significance of this enzyme in the central nervous system is at present largely unknown, the findings reported here must be interpreted with caution.

SUMMARY

1. The action of lysergic acid diethylamide (LSD) on the cholinesterases of man and a number of other animal species is described.

2. LSD is a relatively powerful inhibitor of pseudo-cholinesterase in human serum and brain.

3. The true cholinesterase and tributyrinase of human brain are only slightly affected by concentrations of LSD that cause an almost complete inhibition of pseudo-cholinesterase.

4. Inhibition of pseudo-cholinesterase by LSD is competitive in nature, and is readily reversible.

5. Pseudo-cholinesterase activity of rat, guinea-pig, rabbit, chicken and monkey brain is much less sensitive to inhibition by LSD than is the corresponding enzyme in human brain.

6. LSD is a more powerful inhibitor of pseudo-cholinesterase than are the other ergot alkaloids studied.

We wish to thank Dr. R. T. C. Pratt and Messrs. Sandoz Products Ltd. for kindly providing us with samples of lysergic acid diethylamide and other ergot alkaloids, and Dr. L. C. Thomson for the monkey brains. Our thanks are also due to Mr. R. F. Adams and Mr. D. E. Jarvis for technical assistance, and to the Medical Research Council for grants towards the expenses of this work.

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# CARDIOVASCULAR AND RESPIRATORY ACTIONS OF 5-HYDROXYTRYPTAMINE IN THE CAT

BY

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The respiratory and cardiovascular effects of 5-hydroxytryptamine (HT) in the cat have been described by several authors, but it is still not clear to what extent its effects are due to reflex or to direct actions.

HT causes a temporary arrest of breathing in the cat (Reid and Rand, 1951). Ginzel and Kottegoda (1954) observed that, when small doses (2–6 $\mu$g.) were injected into the carotid sinus region of the cat, there was a brief apnoea followed by stimulation of breathing; these changes were accompanied by a fall of blood pressure. Since denervation of the carotid sinus region abolished these effects they concluded that HT acts on either baro- or chemo-receptors. They also found that, after denervation of the carotid sinus, larger doses of the drug (50–100 $\mu$g.) once again caused similar respiratory and blood-pressure changes. From these and other observations they concluded that the effects produced by large doses of HT were due to actions on the central nervous system. While it is likely that, as with veratridine, large doses of HT cause cardiovascular and respiratory changes by central actions, other mechanisms also participate in the cardiovascular and respiratory effects produced by this substance.

Comroe, Van Lingen, Stroud, and Roncoroni (1953) observed that rapid intravenous injection of HT into cats caused one or all of the following effects: bradycardia, hypotension, apnoea, bronchial constriction, and an increase of right ventricular pressure. They showed that almost all these effects were, or might be, reflex; they concluded that the afferent nerve fibres were mainly in the vagi, and that the sensory receptors for the apnoea, bradycardia, and hypotension lie in the areas supplied by the pulmonary vessels and branches of the ascending aorta. They also suggested that HT might excite the coronary chemo-reflex (Bezold effect). Schneider and Yonkman (1953, 1954) reported that HT stimulates pulmonary stretch receptors, but admitted that some other type of sensory vagal nerve fibres might also be excited. Mott and Paintal (1953), in a small series of experiments, showed that the reflex apnoea caused by HT in the cat arose from sensory receptors lying between the great veins and the left atrium, and that it was abolished by cutting the vagi but not by cooling them to 2–3° C. Mott and Paintal (1953) and Paintal (1954) were unable to find any evidence that this substance stimulates or sensitizes pulmonary stretch, right or left atrial, or depressor, nerve endings. They concluded that the reflex apnoea caused by HT in the cat resembled that for the amidine compounds, and differed from that caused by veratridine; the latter has been shown to stimulate pulmonary stretch fibres (Meier, Bein, and Helmich, 1949; Dawes, Mott, and Widdicombe, 1951; Paintal, 1953). Hence there exists a difference of opinion regarding the site and mode of action of HT in causing reflex apnoea. The present investigation was undertaken in the hope of elucidating this question, and also with a view to analysing further reflex actions of HT in the cat.

## METHODS

The respiratory and cardiovascular changes produced by intravenous injections of HT were studied in 13 cats (1.5–3.2 kg.). The animals were anaesthetized with chloralose (60 mg./kg.) and the respiratory movements recorded by the body plethysmograph (Dawes, Mott, and Widdicombe, 1951). Carotid blood pressure was recorded by a mercury manometer and injections were made into the jugular vein. The cervical vagi were cooled by the method described by Dawes *et al.* (1951). Both vagi were cooled simultaneously to the same temperature.

The effect of intravenous injections of HT on the blood flow in the hind legs was investigated in four cats (2.3–3.4 kg.). These animals were anaesthetized with pentobarbitone (30 mg./kg. intraperitoneally) and heparinized. The aorta was divided in the lower abdomen, a pump unit was introduced between the

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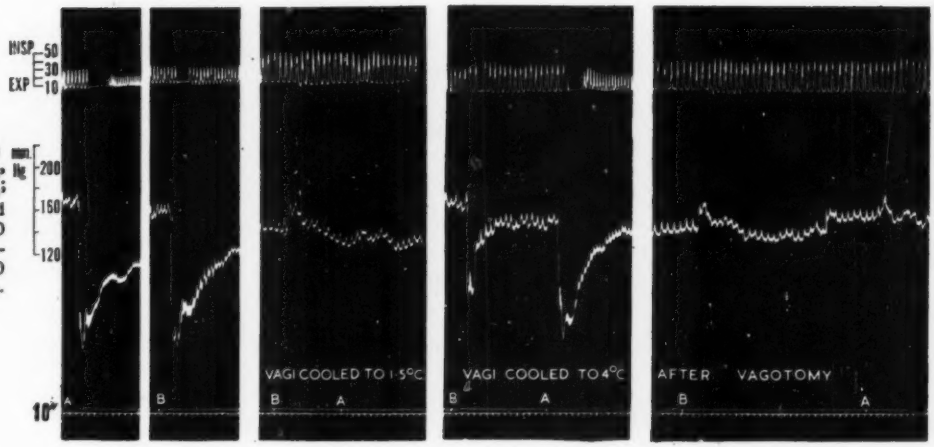


FIG. 1.—Cat. Chloralose 60 mg./kg. Top tracing, respiration (scale in ml.); lower tracing, carotid blood pressure. At A, 50 μg. 2α-naphthyl ethyl isothiourea i.v.; at B, 50 μg. 5-hydroxytryptamine. Time in 10 sec.

cut ends of the aorta (Vane, 1953) and the flow in the hind legs measured by the density flowmeter (Dawes, Mott, and Vane, 1953). The perfusion pressure, which was independent of the mean arterial pressure of the cat, was adjusted to correspond to the cat's blood pressure at the beginning of the experiment. Injections were made into the jugular vein and the carotid blood pressure was measured by a mercury manometer.

The effect of injecting small doses of HT into the heart was studied in 14 cats (2.1–4.6 kg.). They were anaesthetized with chloralose and heparinized. The chest was opened in the midline and the lungs ventilated artificially. Injections were made into the main left coronary artery using the method described by Dawes (1947). Blood pressure was recorded from the carotid artery by a mercury manometer and control injections of the same doses of the drug as were given into the coronary artery were made into a jugular vein.

In all experiments precautions were taken to eliminate errors from the tachyphylaxis which has been reported for HT (Reid, 1952; Freyburger, Graham, Rapport, Seay, Govier, Swoap, and Vander Brook, 1952; Gaddum, 1953; Ginzel and Kottegoda, 1953).

In three cats (2.7–3.8 kg.) anaesthetized with sodium pentobarbitone (32 mg./kg. i.p.) the vagi were divided to exclude reflex phenomena; the action potentials of single nerve fibres in the right vagus arising from slowly adapting pulmonary stretch endings were recorded using the method described by Widdicombe (1954a), but without filters. The intra-tracheal pressure was measured with a condenser manometer attached to one side arm of a tracheal cannula. The other two side tubes were attached to the output and input of a Palmer constant volume respiration pump which provided 20 inflations a minute. The chest was opened so that the pulmonary effects of artificial positive pressure respiration should not be interfered with by spontaneous movements of the chest wall. The tracheal pressure was displayed on one beam of a double-beam cathode-ray oscilloscope and the action potentials on the other. Injections of HT were made through the right brachial vein and the blood pressure was measured in the left femoral or iliac artery by a mercury manometer.

All doses of 5-hydroxytryptamine refer to the creatinine sulphate complex.

## RESULTS

### *The Effect of Vagal Cooling and of Vagotomy on the Cardiovascular and Respiratory Actions of 5-Hydroxytryptamine*

*Circulation.*—In all but 1 of the 13 cats used in these experiments 50–100 μg. HT intravenously caused a fall of blood pressure and bradycardia. Cooling the vagi to 1.5 or 2° C. abolished the effect in 7 cats (Fig. 1). In some of these the effects were not present when the vagi were cooled to 4° C., whereas in others cooling to 6–8° C. also abolished the effects. In 4 cats the effects were not abolished completely by cooling to 4–10° C. (Fig. 2). Out of 10 cats in which vagotomy was performed, in 8 the circulatory

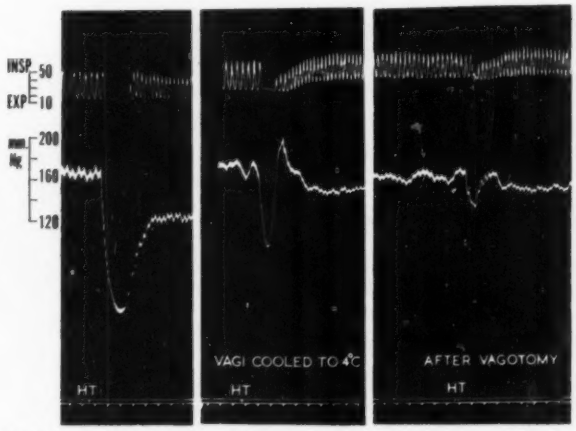


FIG. 2.—Cat. Chloralose, 60 mg./kg. Top tracing, respiration (scale in ml.); lower tracing, carotid blood pressure. At HT, 100 μg. 5-hydroxytryptamine i.v. Time in 10 sec.

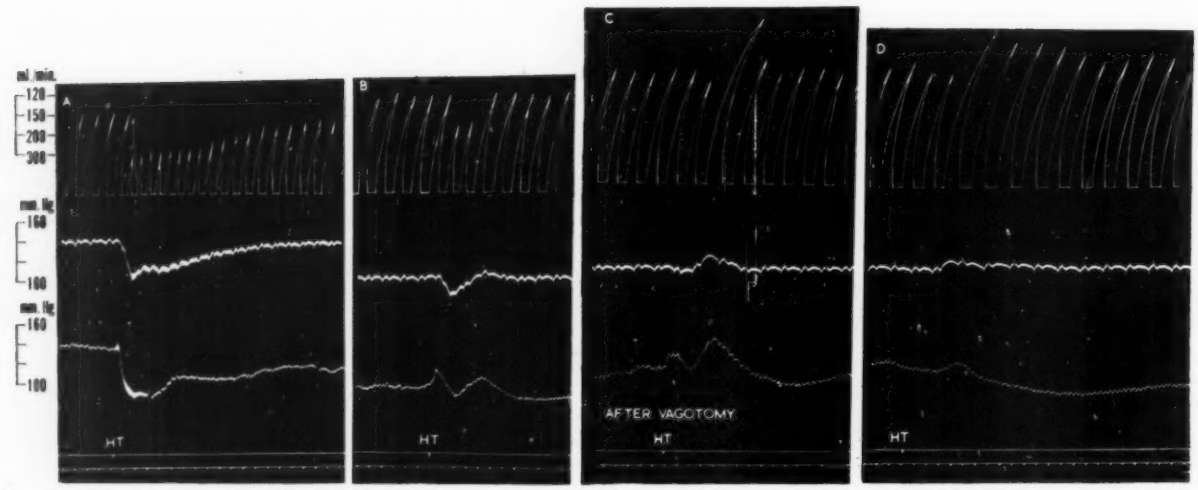


FIG. 3.—Cat. Sodium pentobarbitone, 32 mg./kg. Top tracing, rate of blood flow to perfused hind legs; middle tracing, perfusion pressure; bottom tracing, carotid blood pressure. At HT, 100 μg. 5-hydroxytryptamine intravenously in A, B, and C and intra-arterially in D. Between A and B, 4 mg. of atropine i.v. Between B and C, vagi cut. Time in 10 sec.

effects were abolished and in the other 2 there was still some effect remaining after vagotomy (Fig. 2).

*Blood Flow in the Hind Legs.*—In all 4 cats used in these experiments intravenous injections of 100–150 μg. HT caused an increase in blood flow in the hind legs during the fall of systemic blood pressure. This increase of flow was sometimes followed by a reduction. Comroe *et al.* (1953) believed that the hypotension caused by HT was mainly due to the bradycardia. In our experiments, after the animal was atropinized (1.5 mg./kg.) and the bradycardia prevented, intravenous HT still caused an increase in blood flow in the hind legs though to a less extent than before. Of the 3 experiments in which vagotomy was performed the increase in blood flow was abolished in 2 (Fig. 3), whereas in the third the effect was much reduced both in duration and in magnitude. After vagotomy, intravenous HT (100–150 μg.) often caused only a diminution of flow. When the same doses of the drug were injected into the inflow tube of the flowmeter—a procedure which amounted to direct intra-arterial injection into the hind legs—there was a large decrease in flow. This was probably due to the well-known direct vasoconstrictor action of HT which might normally counteract to some extent the reflex vasodilatation caused by the drug.

*Intra-coronary Injection.*—In all except 1 of the 14 cats used in this series of experiments intra-coronary injection of 5–10 μg. caused a bradycardia and a fall of blood pressure (ranging from 20 to 90 mm. mercury). The same doses intravenously had either no effect on the blood pressure and heart rate or caused a small rise of pressure. Vagotomy was not performed in all these experiments, as the deterioration of the animal's condition—probably hastened by manipulation of the heart and by the effects of the drug—was such that in some experiments it was thought that any results obtained after vagotomy might be equivocal. Of 5 animals in which the vagi were cut

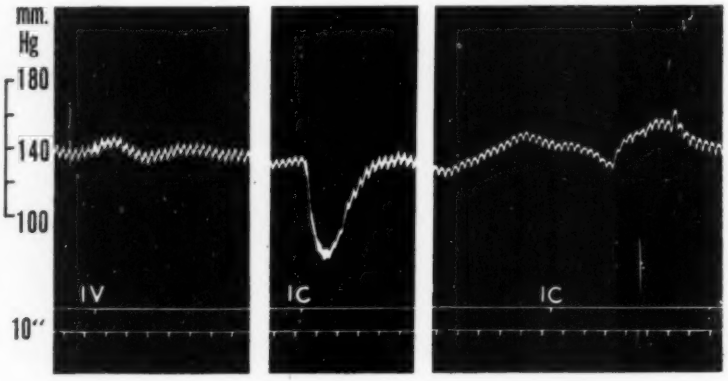


FIG. 4.—Cat. Chloralose, 60 mg./kg. Tracing of carotid blood pressure, 5 μg. HT at signal marks by intravenous (IV) or intra-coronary (IC) injection. Between the second and third portions of record, the vagi were cut. Time in 10 sec.

the hypotension and bradycardia were abolished in 4 (Fig. 4) and greatly reduced in the other. Similarly, in 5 of the 6 cats in which the vagi were cooled the effects were blocked at 6–8° C. In the remaining cat cooling to 6° C. reduced the effects but did not abolish them.

*Respiration.*—In 3 of the 13 cats used in these experiments the respiratory effects caused by injecting 50–100 μg. HT were not clear. In the remaining 10 cats the drug caused a period of apnoea which was often followed by a period of quickened breathing. In several of these animals the latter effect was accompanied by an increase in expiratory volume (functional residual air), as indicated by a rise in the whole record, very similar to that described by Dawes *et al.* (1951) for 2α-naphthyl ethyl isothiourea (see Fig. 2). The respiratory response was not altered by cooling the vagi to 8–10° C.; at temperatures between 8 and 2° C. there was a progressive diminution in the effect. In 5 of 6 cats cooling the vagi to 1.5 or 2° C. abolished the apnoea (Fig. 1) which could again be elicited by injecting HT after the nerves had regained body temperature. In 8 cats the injections were repeated after vagotomy. In 5 of these the apnoea was abolished, whereas in the other 3 there was a small residual effect (Fig. 2). In one cat in which the respiratory action persisted after vagotomy, cooling the vagi to 1° C. had also failed to abolish the apnoea completely.

Thus these observations confirm Mott and Paintal's (1953) conclusion that the effect of small doses of HT on breathing is not abolished by cooling the vagi to temperatures which are known to block conduction in pulmonary stretch fibres and the Hering-Breuer inhibitory reflex (Widdicombe, 1954a and b). Vagotomy always reduces, but does not always abolish, the apnoea ; there are, therefore, at least two mechanisms involved—one operating by means of afferent vagal nerve-fibres which are blocked by cooling below 2° C. (approximately), and the other by non-vagal mechanisms.

*Pulmonary Stretch Receptors.*—As the experiments described above showed that the respiratory response of the cat to HT was certainly not dominated by the Hering-Breuer reflex, it seemed desirable to repeat Schneider and Yonkman's experiments (1953, 1954) from which they concluded that HT stimulated the pulmonary stretch endings. It also seemed important to bear in mind the facts that the activity of pulmonary stretch endings can be modified by drugs known to cause changes in bronchial tone (Widdicombe, 1954c) and that HT can cause direct and reflex broncho-constriction (Comroe, Van Lingen, Stroud, and Roncoroni, 1953).

In three vagotomized cats, under artificial positive-pressure ventilation at constant volume, the action potentials from single pulmonary stretch endings,* and the intra-tracheal pressure, were recorded simultaneously. The intra-tracheal pressure and the frequency of discharge of the nerve fibre were measured at the peak of inflation ; in some experiments the frequency of discharge of the nerve fibre was also measured during deflation.

100–200 μg. HT (twice the quantity needed to produce the reflex effects described above) caused a rise of peak intra-tracheal pressure of from 0.6 to 2.0 mm. Hg (mean 16.5%, range 7–39%) in 14 of 15 tests. The increase in peak intra-tracheal pressure began to be evident on the first or second inflation, 2–7 sec. after the injection, but the maximum effect took 5–13 sec. to develop.

It was therefore clear that the injections of HT caused physical changes in the lung which need to be taken into account when attempting to interpret the effects of the injection on the activity of pulmonary stretch endings. The increase in peak intra-tracheal pressure at constant volume inflation, so regularly observed, is consistent with direct broncho-constriction by the drug.

The effects of the injections of HT on the activity of the pulmonary stretch endings varied. Six fibres showed a clear increase in frequency at the peaks of the inflations after the injection. The maximum increase of frequency (20–104%, mean 42%) usually occurred later (9–22 sec. after the injection) than the maximum pressure change. Three of the fibres showed activity throughout the respiratory cycle, and after the injection their activity increased during the *deflation* periods.

The initial response of six other fibres was a *fall* in frequency (mean 15%, range 6–38%) at the peak of inflations following the injection. In two fibres the peak frequency increased again, while the intra-tracheal pressure remained raised. One fibre which showed a *decrease* in frequency at the peaks of inflation after the injection nevertheless *increased* in frequency during the periods of deflation.

One of the remaining three records showed no rise of intra-tracheal pressure after the injection, and the effect on the peak frequency of the fibre was equivocal. The last two records both showed an increase of intra-tracheal pressure: on one the

* For the purposes of this paper a pulmonary stretch ending is one from which the action potentials increase in frequency during inflation of the lungs and decrease in frequency as they collapse. Endings from several different parts of the tracheo-bronchial tree and lungs may be included (Widdicombe, 1954a).

activity of the fibre showed no change, and on the other the result was equivocal.

It was noticed that aural judgments of the effect of an injection of HT on the activity of pulmonary stretch ending were unreliable, since the ear tended to confuse an increase of activity during the deflationary part of the cycle with a total increase of frequency; in such circumstances a decrease of frequency at the peak of inflation may not be appreciated.

## Discussion

*Circulation.*—The cardiovascular effects of 5-hydroxytryptamine are complex, but it is clear that a proportion of the bradycardia and hypotension caused by the intravenous injection of 50–100 μg. is due to the coronary chemo-reflex (Dawes and Comroe, 1954), which can also be elicited by the intra-coronary injection of 5–10 μg. of HT. In most cats the cardiovascular effects of the intra-coronary injections were abolished by cooling the vagi to 6–8° C. At this temperature some, but not many, of the vagal *efferent* nerve fibres are still functional (Dawes, Mott, and Widdicombe, 1951), and if the *afferent* nerve fibres were intact there would be peripheral vasodilatation, which is shown, by the experiments on the hind legs, to contribute to the fall of blood pressure. The temperature at which the coronary chemo-reflex is abolished is a little lower than that reported for veratridine, and possibly lower than that for the amidines (Dawes, Mott, and Widdicombe, 1951), though the situation with these compounds is confused, as they also excite the pulmonary depressor chemo-reflex (Dawes and Comroe, 1953). HT also probably excites the pulmonary depressor reflex (Comroe, Van Lingen, Stroud, and Roncoroni, 1953), and this makes more difficult the precise definition of the coronary chemo-reflex with this compound. It is, however, clear, from the experiments reported in this paper, that the vagal temperatures required to abolish the whole of the vagal depressor effects are generally lower than those required to abolish only that portion due to the stimulation of receptors in the distribution of the coronary artery. This is indirect evidence in support of the stimulation of the pulmonary depressor reflex by HT in cats.

*Respiration.*—The results described in this paper confirm those of Mott and Paintal (1953). The apnoea caused by the injection of 50–100 μg. HT creatinine sulphate into cats can, in most instances, be shown to be reflex, since it is abolished by vagotomy or by cooling the vagi to 1–2° C. In some cats such injections cause an apnoea of non-vagal origin; but this, of course, does not exclude the co-existence of the reflex apnoea in the intact animal.

The experiments in which the vagi were cooled offer no support for the view that the pulmonary stretch endings are concerned to a significant extent in the reflex apnoea, since conduction along the fibres from such endings is abolished at 8°–10° C. (Dawes *et al.*, 1951) and cooling the vagi to this temperature does not reduce the respiratory response to HT.

The experiments also show that injection of 100–200 μg. HT (which is double the quantity required to produce the reflex apnoea) is sufficient to cause a mean increase of 16.5% in intra-tracheal pressure when the lungs are inflated at constant volume. This must mean that the injection causes a change in the compliance of the lungs. Two possible causes of this are broncho-constriction (Comroe, Van Lingen, Stroud, and Roncoroni), or a change in the amount of distribution of blood in the lungs —perhaps as a result of pulmonary vasoconstriction (Ginzel and Kottegoda, 1953). Whatever be the cause of the change of the physical response of the lung to constant volume inflation after HT, it is obvious that the effect of this drug on the activity of the pulmonary stretch fibres cannot be properly interpreted unless the disposition of the pulmonary stretch endings in the lung is known. The evidence on this point is somewhat indirect, though it has been suggested that these endings are in the bronchi, bronchioles or smaller air passages (Widdicombe, 1954c). If this view is correct, any drug which causes a change in bronchial tone may be expected to affect indirectly stretch receptors in the bronchial muscle. The effect on any individual receptor would depend on whether it was proximal or distal to the constricted segment, and whether it was in series or in parallel with the muscular elements. In the absence of histological or other evidence of the location of the endings further comment would be speculative, but it is at least possible that a drug could consistently cause broncho-constriction and yet have variable effects on the pulmonary stretch endings, according to their position relative to the constriction, and to the amount and distribution of the constriction.

If the twelve pulmonary stretch endings which gave clear-cut responses to injections of HT were a fair sample of such endings, it may be concluded that the total number of impulses due to non-reflex stimulation arriving at the respiratory centre would not be greatly increased, since the activity of six of the fibres increased whereas that of six

decreased. It is, of course, possible that the endings whose activity decreased are of a different type from those whose activity increased (Widdicombe, 1954a).

Veratridine is a substance which does cause reflex apnoea by stimulation of the pulmonary stretch endings (Dawes, Mott, and Widdicombe, 1951), but the response is much more intense than we have observed with 100–200 μg. of HT. The discharges of an ending stimulated with veratridine give rise to a whine in the loud-speaker within a very few seconds of the injection, and the latency of the reflex apnoea (as measured by the inhibition of phrenic motor neurones) is only 3.6–5.9 sec. (Jones, 1952). The response of a pulmonary stretch ending to HT takes 9–22 sec. to reach its peak and usually follows, rather than precedes, the increase in tracheal pressure. Presumably the smaller dose required to elicit the reflex respiratory response would cause even less disturbance of the pulmonary stretch endings.

Schneider and Yonkman (1953) have recorded stimulation of the pulmonary stretch endings with HT as intense as that observed by Dawes, Mott, and Widdicombe (1951) with veratridine, but the latency is still very long (5–15 sec.). It remains to be explained why a greater degree of stimulation was observed by Schneider and Yonkman (1953, 1954) than in the experiments described in this paper.

There was probably not any important difference in the depth of anaesthesia or in the dose of HT used in the two sets of experiments ; but it is conceivable that the degree of smooth muscle tone was not identical, and this may influence the bronchial responses (Widdicombe, 1954c). Inspection of Schneider and Yonkman's published records does, however, show that conspicuous bradycardia occurred. The presumption is that the second vagus was intact and that the coronary chemoreflex and the pulmonary depressor reflex (Dawes and Comroe, 1953) were active. The reflex respiratory affects of HT include broncho-constriction (Comroe, Van Lingen, Stroud, and Roncoroni, 1953). It is therefore likely that reflex as well as direct broncho-constriction occurred in Schneider and Yonkman's cats, and this might well have led to greater indirect stimulation of the pulmonary stretch endings. Paintal (1954) has recently suggested a similar interpretation of Schneider and Yonkman's observations. It is also possible that the reflex cardiac slowing might have caused an alteration in the volume or distribution of the blood in the lungs, but investigations on this point are lacking (Dawes and Comroe, 1953). Circulatory factors may be unimportant, since Schneider and Yonkman (1953) prevented the bradycardia with " Pendiomide " (Ciba 9295), but still obtained an increase in the activity of the pulmonary stretch endings. Schneider and Yonkman (1954) were unable to cause much increase of activity of pulmonary stretch endings by injecting HT in the rabbit, a species in which it is sometimes rather difficult to cause broncho-constriction (Banister, Fegler, and Hebb, 1950).

In conclusion, it seems certain that, in cats, HT causes a reflex apnoea mediated by small afferent nerve fibres, as yet unidentified, in the vagus. This apnoea closely resembles that caused by the amidines but is complicated by additional factors. It seems rather unlikely that HT stimulates directly the pulmonary stretch endings. However, the direct and reflex broncho-constriction which this compound is known to cause may contribute, by indirect stimulation of the pulmonary stretch endings, to the reflex apnoea observed, but not to such a degree as to permit any diminution in the total reflex respiratory response when the vagi are cooled to 8–10° C.

## Summary

1. In 7 of 13 cats the bradycardia and fall of blood pressure caused by 50–100 μg. 5-hydroxytryptamine (HT) intravenously was abolished by cooling the vagi to 1.5 or 2° C. or by vagotomy. The hypotension was accompanied by increased blood flow through the hind legs, not abolished by atropine but greatly reduced or abolished by vagal section. HT thus causes bradycardia and a fall of blood pressure by stimulating afferent vagal fibres ; reflex peripheral vasodilatation contributes to the response.

2. Intra-coronary injection of 5–10 μg. HT caused bradycardia and hypotension (14/15 cats), usually abolished by cooling the vagi to 6–8° C. or by vagotomy. This accounts for part of the reflex cardiovascular effects caused by 50–100 μg. HT intravenously.

3. The apnoea elicited by HT (50–100 μg. i.v.) was usually abolished by cooling the vagi to 1.5 or 2° C. or by vagotomy and was therefore reflex ; but in some cats a brief apnoea was still produced by HT after vagotomy.

4. Intravenous injection of 100–200 μg. HT caused a small increase in peak intra-tracheal pressure in vagotomized cats given artificial positive-pressure ventilation at constant stroke volume, and was followed by changes in the rate of discharge of pulmonary stretch fibres. It is suggested that

these changes were secondary to direct bronchoconstriction caused by the HT.

5. The differences between the effects of HT on the activity of pulmonary stretch endings as described in this paper and as described by Schneider and Yonkman (1953, 1954) are discussed.

6. It is concluded that stimulation of pulmonary stretch endings is unlikely to play a dominant role in the production of reflex apnoea by the intravenous injection of 50–100 $\mu$g. HT in the cat.

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# ANTITUBERCULOUS EFFECTS OF CERTAIN SURFACE-ACTIVE POLYOXYETHYLENE ETHERS

BY

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We have previously reported (Cornforth, Hart, Rees, and Stock, 1951) that a commercial surface-active agent, the non-ionic "Triton A20" (Rohm and Haas Co., Philadelphia),* has a suppressive effect on experimental tuberculosis in the mouse, and that the effect is shared by a number of similarly constituted products prepared by ourselves. These observations introduced a new type of antituberculous agent. Furthermore, since the therapeutically active products failed to inhibit the multiplication of tubercle bacilli *in vitro*, it was suggested that they might in some way modify the host's response to a tuberculous infection (Rees, 1953). The antituberculous properties of Triton A20 have been confirmed by Solotorovsky and Gregory (1952), who also reported a synergistic action with dihydrostreptomycin in a mouse infection. In addition to suppressing acute tuberculosis in mice, these agents can produce appreciable regression and healing of an established infection in the guinea-pig (Rees, 1953). The present paper supplies necessary details of the earlier work and describes further developments. Some of the results were briefly communicated by Cornforth, Hart, Nicholls, and Rees (1953).

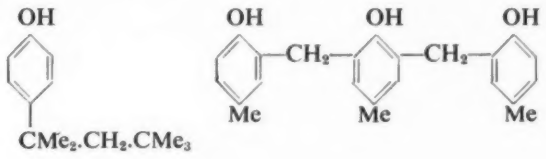
## CHEMICAL SECTION

### Chemical Nature of Products

Our first experiments, which were based on a chemical examination of Triton A20, and which resembled procedures given in U.S. Patent No. 2,454,541, involved acid-catalysed condensation of *p*-(1:1:3:3-tetramethylbutyl)phenol (I; referred to henceforward as octylphenol) with formaldehyde, followed by heat treatment of the product and reaction of the resulting resin with 17 to 20 molecules of ethylene oxide for each phenolic group. The final product, a water-soluble syrup, differed slightly in some of its properties from Triton A20, but proved equally effective against experimental tuberculosis in the mouse.

* The solute, of which Triton A20 is an aqueous solution, is known as WR1339

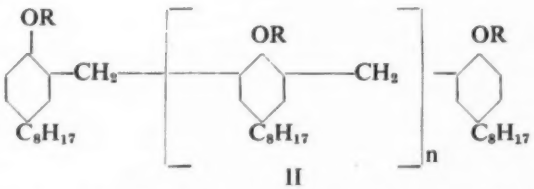
According to the patent cited above, a product thus prepared should have a general formula (II) and the octylphenol-formaldehyde condensation product should be (II; R = H). There is no reason to doubt




I




III




II

(R=$(CH_2CH_2O)_xH$, except where otherwise stated.)

the essential truth of this contention. The composition of our octylphenol-formaldehyde condensation product agreed with the expression (II; R = H). Though the product was insoluble in aqueous alkali, free phenolic groups could be demonstrated by the tautomeric shift in ultra-violet light absorption when alkali was added to an alcoholic solution. Finally, the acid-catalysed formation of linear polymers corresponding to formula (II; R = H) was shown, with *p*-cresol, by Megson and Drummond (1930), who isolated a tricyclic compound (III) from the reaction mixture.

However, the condensation product of octylphenol with formaldehyde satisfies none of the ordinary criteria of purity. The antituberculous activity observed after condensation with ethylene oxide might, it was thought, be due to substances of different structure present in relatively small proportion. Even if the activity was indeed due to molecules of the general formula (II), it might still be associated with a particular value, or range of values, of *n*.

Under appropriate conditions, reaction of *p*-substituted phenols with formaldehyde can certainly lead

to products not expressible by formula (II ; R = H) ; for example, substances in which aromatic nuclei are joined by a dimethylene ether link, - $CH_2$ - O - $CH_2$ - , instead of the methylene link, - $CH_2$ -. Such substances are reported to be unstable to heat, and to afford benzyl bromides on treatment with hydrogen bromide in benzene (von Euler, Adler, Cedwall, and Törngren, 1942 ; Zinke, Tomio, and Lercher, 1942). Our products had been heated strongly during preparation, and they acquired no organically bound bromine on treatment with hydrogen bromide. Hence it is unlikely that ether linkages of this sort are present, and the same applies to hydroxymethyl groups, - $CH_2OH$. Yet the decomposition by heat of such *o*-hydroxybenzyl ethers or *o*-hydroxybenzyl alcohols can give rise to products among which chromans, diphenylethylenes, and diphenylethanes have been mentioned (Alder, von Euler, and Cedwall, 1942 ; Hultzsch, 1950). The possible presence of such substances in minor amount is more difficult to exclude except on the general ground that benzyl alcohols and benzyl ethers are unlikely to attain significant concentrations in an acid-catalysed condensation. These considerations made it very desirable to prepare, for condensation with ethylene oxide, pure substances of known structure.

Preparation of a series of substances having the structure (II ; R = H) in which $n$ is 0, 1, 2 . . . , was an early objective : the parent of this homologous series is of course octylphenol (I) itself, which on condensation with ethylene oxide gave products of the general formula (IV). The dicyclic compound ($n$ = 0), was easily obtained from formaldehyde and an excess of octylphenol. For the tricyclic compound ($n$ = 1), 2:6-bishydroxymethyl-4-*tert.*-octylphenol (V) was required ; this has been made before (Sherman, 1938 ; U.S. Patent 2,488,134), but our own procedure is given in the experimental section. Two crystalline modifications of the substance were encountered.

Condensation of *p*-alkylphenols with substances of type (V) has been reported (Niederl and Ruderman, 1945) to give polymers higher than the tricyclic stage. This is true, and might have been expected, when equivalent quantities of the reactants are employed ; but when the bishydroxymethylphenol (V) was heated

IV V

with an excess of octylphenol and a little trichloroacetic acid, the desired tricyclic substance (II ; R = H ; $n$ = 1) was obtained without difficulty. It was isolated by distillation in high vacuum and was later obtained crystalline. Similar condensations of *p*-cresol with its bishydroxymethyl derivative have been described (Megson and Drummond, 1930 ; Koebner, 1933), hydrochloric acid being the catalyst. A trial of this catalyst gave a substantial amount of dicyclic material (II ; R = H ; $n$ = 0), the strong acid evidently causing fission of a hydroxymethyl group.

Serious difficulties were met in attempts to make the pure tetracyclic compound (II ; R = H ; $n$ = 2). A modification of Koebner's (1933) technique was used, excess of the dicyclic phenol (II ; R = H ; $n$ = 0) being condensed with formaldehyde in acetic acid with hydrogen chloride. Excess of dicyclic phenol was removed by distillation ; short-path distillation in a molecular still then gave a preparation containing the desired product. This could not be crystallized. To obtain pure material, counter-current distribution was first tried ; the solvent pair was ligroin—94% ethanol. Though this operation provided evidence that the product of two successive molecular distillations was not homogeneous, material having a constant partition ratio was not obtained even after repeated distributions.

The pentacyclic compound (II ; R = H ; $n$ = 3) was obtained by condensation of 2:6-bishydroxymethyl-4-*tert.*-octylphenol (V) with excess of dicyclic compound. The crude product was partially purified by molecular distillation.

Reversed-phase partition chromatography of the various condensation products described above was now examined, primarily as a method of testing the homogeneity of different preparations. Procedures were found for separation of the tetracyclic and pentacyclic phenols from each other and from lower homologues. It was shown that the tetracyclic compound was contaminated with lower homologues even after two molecular distillations and two counter-current distributions. The pentacyclic compound (undistilled) was similarly contaminated, and the tetracyclic compound was one of the impurities. Evidently this was formed by fission of a hydroxymethyl group, the resulting formaldehyde then coupling two molecules of dicyclic compound.

In principle, these separations on the milligram scale could have been applied to prepare larger amounts of pure materials for reaction with ethylene oxide ; but at this time we were attracted by another possibility. Zinke and Ziegler (1944) described a crystalline product, obtainable under special conditions from formaldehyde and *p-tert.*-butylphenol, to which a macrocyclic structure (VI ; R = $CMe_3$) was assigned. A later paper (Zinke, Zigeuner, Hössinger, and Hoffmann, 1948) reported, without characterization, similar products from certain other phenols. Repetition of the work on *p-tert.*-butylphenol demonstrated that two compounds could be separated from the crude crystalline product. Further, when the process was applied to octylphenol, two crystalline compounds were again isolated.

The two compounds from *p-tert.*-butylphenol were crystalline, high-melting, and sparingly soluble in most solvents. Analysis indicated for each an empirical formula $(C_{11}H_{14}O)_n$. It followed that the oxygen in the molecule all originated from *p-tert.*-butylphenol, and the preparation of crystalline acetates $(C_{11}H_{13}OAc)_n$ showed that all oxygen atoms were still

in the form of phenolic hydroxyl groups. The presence of a cyclic structure analogous to (VI), rather than a linear structure of type (II ; R = H), was deducible from chemical evidence indicating the absence of a free position *ortho* to a hydroxyl group : such positions are present at each end of a linear polymer (II ; R = H), but are absent from a cyclic polymer. Whereas linear polymers (II ; R = H) reacted readily with a chloroform solution of bromine or—in strongly alkaline solution—with *p*-nitrobenzenediazonium chloride, these two substances were inert to both reagents, though coupling with the diazo reagent did take place under conditions known to cause fission of methylene bridges in the *o*- or *p*-position (Ziegler, 1951). The two compounds from octylphenol were similar in chemical properties to their *tetrt.*-butyl analogues.

If a macrocyclic structure is accepted for these substances it still remains to determine the number of phenolic units participating in the large ring. With the compounds from *p-tert.*-butylphenol, consistent values could be obtained by the Rast method, for the molecular weight of the lower-melting substance (hereafter called LBC : low-melting butylphenol cyclic compound), and of the acetate of HBC, the higher-melting substance (itself too insoluble to use). For both HBC and LBC the structure (VI ; R = $CMe_3$), containing four phenolic units and four methylene bridges, was indicated.

The Rast method was less satisfactory with acetates of the two compounds from octylphenol (HOC and LOC). Results were not consistent and were lower than expected. We therefore submitted specimens of HOC, LOC, HBC, LBC, and HOC acetate for *x*-ray crystallographic examination to Dr. D. M. C. Hodgkin, F.R.S., whose valuable assistance is greatly appreciated. Her report is:

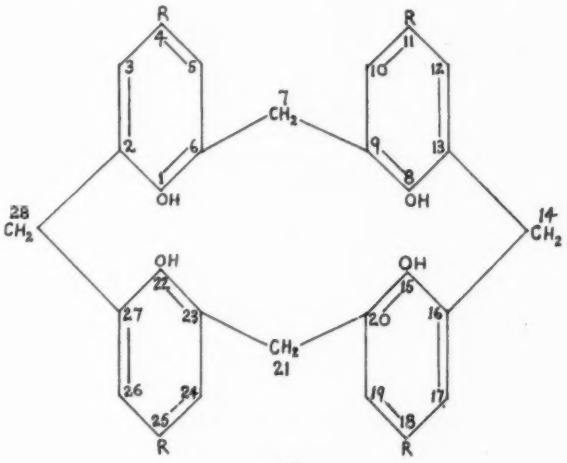
"The preliminary crystallographic data on HOC, LOC, HBC, LBC, and HOC acetate, obtained by E. K. Hunter and M. Mackay, are summarized in Table I. Both HOC and HBC have very complex crystal structures in which the asymmetric units have respectively four and three times the weight required for a tetrapolymer. Formally they admit several solutions for the molecular complexity of these compounds. HOC acetate is rather simpler; the molecule here from the *x*-ray evidence is most probably a 4-polymer (space group $P\bar{1}$), but might be either an 8-polymer, or possibly a 7-polymer (P1), since difficulty was experienced in measuring accurately the lattice constants of the triclinic crystals.

"LOC and LBC both have crystal structures which indicate they are tetrapolymers. Formally, in the case of LOC, the molecule might correspond with a twofold polymer or with an eightfold polymer having a centre of symmetry in the molecule. The latter is, however, not a stereochemically probable solution, and the molecule here almost certainly corresponds with the crystal asymmetric unit. LBC crystallizes in the tetragonal system; here the molecule is proved not only to be a fourfold polymer but also to have either a fourfold or a fourfold alternating axis of symmetry."

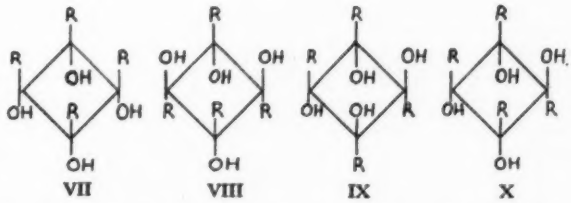
Though they do not establish with certainty the molecular complexity of all the compounds the crystallographic data are consistent with the view that all four condensation products have the general formula (VI). This is reinforced by the Rast molecular weight determinations in the *tert.*-butyl series ; those in the *tert.*-octyl series at least refute the possibility, admitted by the crystallographic data, of molecules containing eight phenolic nuclei. We therefore conclude that HOC–LOC and HBC–LBC are pairs of stereoisomerides.

Examination of models indicates that molecules of structure (VI) may assume four different, not readily interconvertible, configurations. These are illustrated diagrammatically (VII to X). Stereoisomerism is possible here because the phenolic nuclei cannot rotate about the bonds joining them to the methylene groups. The molecular symmetry found crystallographically for LBC establishes that this particular molecule has either configuration VII or VIII. It is interesting that LBC forms a clathrate compound with chloroform, from which the solvent is not expelled even after prolonged heating *in vacuo*.

The name *cyclo*tetra-*m*-benzylene is suggested for the ring system with numbering as shown (VI).



VI



VII VIII IX X

*Reaction of the Phenolic Products with Ethylene Oxide*

Addition of ethylene oxide to the various phenolic products described above was carried out under essentially similar conditions. The product in benzene or toluene, after addition of a little sodium hydroxide as catalyst, was heated with ethylene oxide in a stainless-steel autoclave of 100 ml. capacity. Heating was continued until the ethylene oxide was consumed.

It is highly probable (see, e.g., Miller, Bann, and Thrower, 1950) that the extension of a polyoxyethylene chain under alkaline catalysis involves (1) ionization of the terminal alcoholic group by the alkali, and (2) reaction of the resulting anion with another molecule of ethylene oxide. A phenoxide ion being more easily formed than an alkoxide ion, it may be supposed that phenolic groups will react in preference to alcoholic groups. This is borne out by an experiment in which HOC was heated with ethylene oxide in the presence of a relatively mild base, diethylamine. The product was crystalline, and analysis indicated that reaction under these conditions had ceased after addition of one molecule of ethylene oxide to each phenolic group : that is, the tetra-$\beta$-hydroxyethyl ether of HOC was formed. It may therefore be concluded that no free phenolic group remained in our preparations after condensation in the usual manner with ethylene oxide.

The final products (e.g., HOC-20) are often given a number denoting the average number of ethylene oxide units attached to each phenolic group. This figure is an over-all one, based on the consumption of ethylene oxide ; but it is at least possible that some polymerization of ethylene oxide may be initiated by hydroxyl ions as well as by phenoxide ions. Inert polyethylene glycols would then form part of the product and the average length of the chains attached to phenolic residues would be correspondingly shorter. This possibility cannot be excluded as yet. Again, the value given for the chain-lengths is only an average. Individual chains may range rather widely on either side of the mean, and the statistical distribution may vary in different preparations of the same average chain-length. Though these reservations must be made, the "average chain-length" is a useful figure allowing comparison of one preparation with another.

## Experimental Procedures

*Preparation of Octylphenol-Formaldehyde Polymers.*—Recrystallized octylphenol (61.8 g. ; m.p. 83–84°) and hydrated oxalic acid (37.0 g.) were melted together. Formaldehyde (23.1 ml. of 39% w/v aqueous solution) and water (4 ml.) were added and the mixture was stirred and boiled gently under reflux. After 6 hr. toluene (70 ml.) and water (30 ml.) were added ; 10 min. later, heating and stirring were discontinued. Next day the upper layer was separated and the lower layer stirred with a little more toluene. The toluene solution was washed with saturated aqueous $NaHCO_3$ and then with water. The solution was transferred to a distillation flask placed in an oil bath. Solvent was removed at low pressure (water pump), the bath temperature being gradually raised to 160°. Nitrogen was then passed through the capillary leak and the temperature was raised slowly to 220°. Finally the product was heated at 245–255° (air bath) for 1–1¼ hr. at 1–1.5 mm. pressure (nitrogen). The residue (59.2 g.) cooled under nitrogen to a clear, pale amber, brittle resin ($D_2$ resin). (Found : C, 82.6 ; H, 10.0. Calc. for an infinite linear polymer (II ; R = H ; $n = \infty$) : C, 82.6 ; H, 10.1%).

A resin depleted of lower homologues was prepared by heating $D_2$ resin (2.97 g.) at 0.01 mm. pressure in an air bath rising rapidly to 320°. To reduce pyrolysis, the time of heating above 280° was kept short (15 min.). The residue (1.9 g.) was an amber resin ($D_2A$ resin).

2:2′ - *Dihydroxy* - 5:5′ - *di*(1:1:3:3-*tetramethyl* - *butyl*) - *diphenyl* - *methane* (II ; R = H ; $n = 0$).—Octylphenol (37.2 g. ; 4 equiv.), 40% w/v aqueous formaldehyde (3.40 ml. ; 1 equiv.) and hydrated oxalic acid (22.0 g.) were stirred under reflux in a bath at 120–130° for 1 hr., then at 110–115° for a further 5 hr. Toluene (40 ml.) and water (20 ml.) were added, and refluxing and stirring were continued for 45 min. Next day the toluene solution was separated, washed neutral with water, and evaporated at low pressure. Excess octylphenol was removed by distillation at 0.5 mm. from a bath at 200° ; the product (12.3 g. ; 64%), a pale yellow viscous oil, then distilled as the bath temperature was raised to 280°. Crystallization of the distillate from light petroleum then gave the *dicyclic phenol* as colourless prisms, m.p. 152–153° (Found : C, 82.3 ; H, 10.0 ; M(Rast), 450. $C_{29}H_{44}O_2$ requires C, 82.1 ; H, 10.4% : M, 424).

2:6-*Bishydroxymethyl* - 4 - (1:1:3:3 - *tetramethylbutyl*) - *phenol* (V).—Octylphenol (15.0 g. ; m.p. 84°), aqueous formaldehyde (150 ml. of 40% w/v) and a solution of NaOH (4.5 g.) in water (18 ml.) were shaken together and kept at room temperature for a week. The filtered solution was diluted to 350 ml., acidified with acetic acid, and extracted with ether (3 × 100 ml.). The ether extracts were washed successively with water, dilute $NaHSO_3$, and water ; filtered, and concentrated. The residual violet gum was dissolved in methanol (15 ml.). Water (15 ml.) and light petroleum (b.p. 60–80° ; 15 ml.) were added and the mixture, after shaking, was left at 0°. Crystallization was best allowed to proceed for a long time (two months). The clustered colourless rhombs were collected and recrystallized from light petroleum (b.p. 60–80°) giving the *bishydroxymethylphenol* (9.95 g.) in the stable needle form, m.p. 76–77°. A specimen was analysed after three further crystallizations and drying over phosphoric oxide (Found : C, 72.6 ; H, 9.7. Calc. for $C_{16}H_{26}O_3$ : C, 72.2 ; H, 9.8%). The unstable rhombic form, m.p. 71°, could be obtained by allowing a dilute solution in light petroleum to stand for a few days in a lightly corked flask. This form is apparently the one obtained previously (Sherman, 1938).

2:6 - *Di*[2 - *hydroxy* - 5 - (1:1:3:3 - *tetramethyl* - *butyl*) - *benzyl*] - 4 - (1:1:3:3 - *tetramethylbutyl*) - *phenol* (II ; R = H ; $n = 1$).—The above bishydroxymethylphenol (5.01 g.), octylphenol (15.6 g.), and trichloroacetic acid (0.6 g.) were melted, shaken thoroughly, and heated at 90–100° (bath) for 3½ hr. Excess octylphenol was removed by distillation up to 200°/0.01 mm. and the residue (in three batches) was distilled from bulbs at 300–330° (air bath)/0.01 mm.,

a small fore-run of octylphenol being rejected. The clear pale yellow distillate (7.95 g.) was redistilled at 300° (bath)/0.005 mm.; on cooling it formed a brittle almost colourless resin (Found : C, 82.0; H, 10.4; M (cryoscopic in phenol), 629, 614, 625. $C_{44}H_{66}O_8$ requires C, 82.2; H, 10.3%; M, 642). This *tricyclic phenol* (II; R = H; $n$ = 1) eventually crystallized from methanol; after recrystallization twice from methanol-water and once from methanol it formed colourless needles, m.p. 95–98° (clear at 105°). The analytical sample was dried over phosphoric oxide *in vacuo* (Found : C, 82.3; H, 10.0%).

*Crude Tetracyclic Phenol* (II; R = H; $n$ = 2).—The dicyclic phenol (5.43 g.; prepared as above) was dissolved in acetic acid (50 ml.), and formaldehyde (0.245 ml. of 40% w/v) was added. The clear solution was saturated with dry HCl. After four days the mixture was poured into water (500 ml.) and the turbid liquid was extracted with light petroleum (b.p. 60–80°). The petrol solution was washed with water and evaporated. The product was distilled in bulbs up to 260° (bath)/0.01–0.05 mm., to remove most of the dicyclic phenol. The residue (2.70 g.) was a pale yellow resin ($D_{10}$ resin).

*Crude Pentacyclic Phenol* (II; R = H; $n$ = 3).—The dicyclic phenol (4.78 g.), the bishydroxymethylphenol (0.75 g.), and trichloroacetic acid (0.3 g.) were dissolved in acetic acid (30 ml.). The solution after being heated at 90–100° for 5½ hr. was left for several days at room temperature. The mixture was then worked up as described in the previous experiment; the residue (3.03 g.) after removal of excess dicyclic phenol was a pale yellow resin having a greenish fluorescence.

*Molecular Distillation of Crude Tetracyclic and Pentacyclic Phenols.*—The still was based on the design of Smith and Matalon (1950). In one experiment crude tetracyclic phenol ($D_{10}$ resin; 7.62 g.) was distilled in two batches. After a few minutes at 280°/0.002 mm. the still was cooled and a crust of dicyclic phenol and splashed material were washed off the condenser. The distillation (at 270–290°) was then continued for 2¼ hr. The distillate (2.53 g.) was redistilled (3¼ hr. at 250–260°/0.001 mm.) giving a brittle amber resin ($D_{10}$A resin). The residue in the still from the first distillation is termed $D_{10}$B resin.

The crude pentacyclic phenol (2.74 g.) was distilled similarly at 300–310°/0.002 mm. for 2¼ hr., then 3 hr. The combined distillate (0.3 + 0.1 g.) formed a clear amber resin with a green fluorescence ($D_{12}$ resin).

*Countercurrent Distribution of Crude Tetracyclic Resins $D_{10}$ and $D_{10}$A.*—A 12-tube all-glass Craig apparatus (Craig, 1950) was used. The solvents were 94% v/v aqueous ethanol and ligroin (b.p. 88–101°) mutually saturated at the cold-room temperature (1° ± 1°) at which the distributions were effected. The $D_{10}$ resin (1.14 g.) was dissolved in the first upper layer and the solution was passed in the known manner through the apparatus, lower layer volumes being 92 ml. and upper layers 20 ml. The process was stopped when the original upper layer had been equilibrated in the 12th and last tube. The ultraviolet absorption at 287 mμ of each upper and lower layer was determined after suitable dilution. Thereafter, the total contents of the first five tubes were combined and the solute (0.64 g.) redistributed as before, but with an upper layer volume of 30 ml. The solute (0.43 g.) from the first five tubes was then redistributed, using an upper layer volume of 50 ml. The results of these three distributions are shown in Figs. 1, 2, and 3. Two successive distributions were carried out in a similar manner with $D_{10}$A resin (Figs. 4 and 5). It is evident, even with the final distributions, that the partition ratio varies continuously from tube to tube. (The implicit assumption that optical

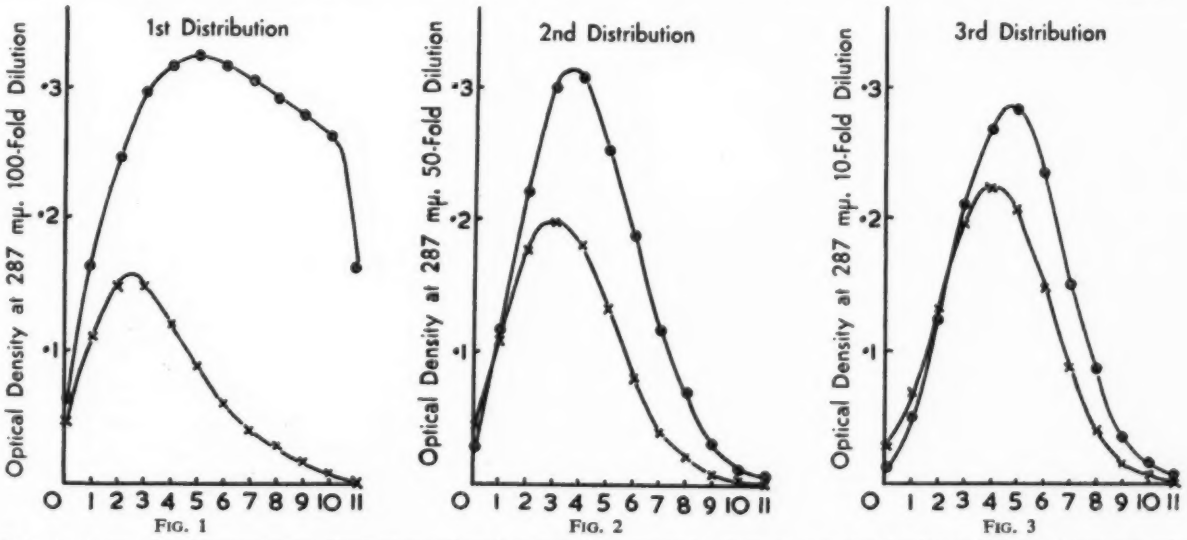

1st Distribution
2nd Distribution
3rd Distribution
Optical Density at 287 mμ. 100-Fold Dilution
Optical Density at 287 mμ. 50-Fold Dilution
Optical Density at 287 mμ. 10-Fold Dilution
·1
·2
·3
0 1 2 3 4 5 6 7 8 9 10 11

FIG. 1 FIG. 2 FIG. 3

FIGS. 1, 2, and 3.—Countercurrent distributions of crude tetracyclic compound. Ordinates: optical density at 287 mμ. Abscissae: tube number. ○, upper layers; ×, lower layers.

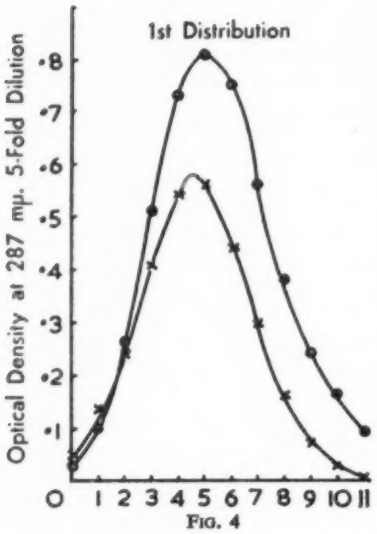



FIG. 4

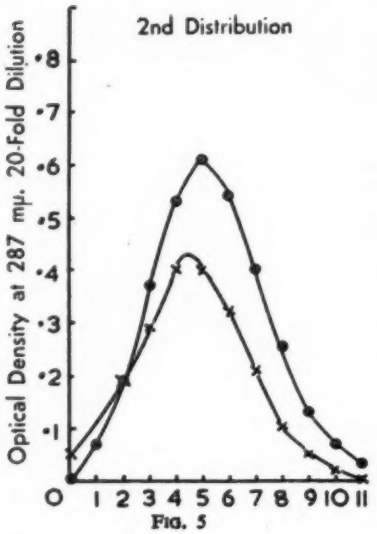



FIG. 5

FIGS. 4 and 5.—Countercurrent distributions of distilled tetracyclic compound. Ordinates: optical density at 287 mμ. Abscissae: tube number. ○, upper layers; ×, lower layers.

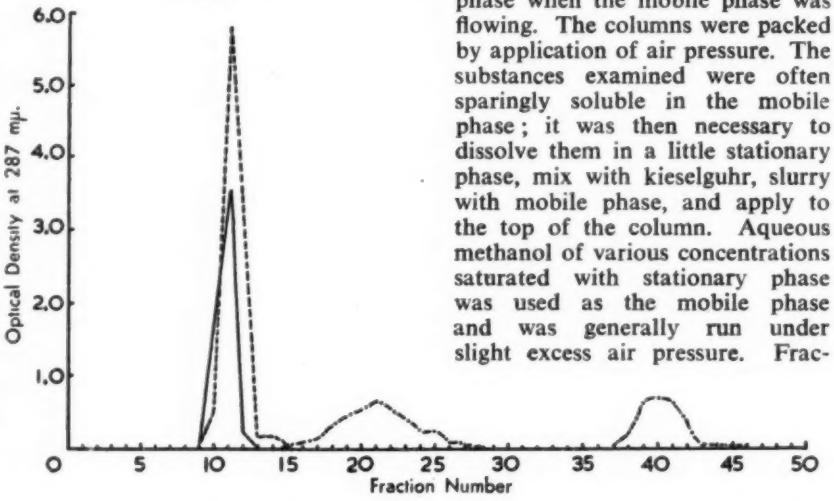



FIG. 6.—Partition chromatography of dicyclic and tricyclic compounds. Ordinate: optical density at 287 mμ. Abscissa: fraction number. ——— Dicyclic (2 mg.); silane-treated hyflo (12.5 g.); *n*-octane (2.0 ml.); 92.5% v/v aq. methanol. – – – – Tricyclic (3 mg.); silane-treated hyflo (12.5 g.); *n*-octane (2.0 ml.); 92.5% v/v aq. methanol. –·–·–·– Dicyclic (1.5 mg.)+tricyclic (1.0 mg.); silane-treated hyflo (6.25 g.); $C_{12}$ hydrocarbon (5.0 ml.); 70% v/v aq. methanol changed to 96% after collecting fraction 34.

density is proportional to concentration, i.e., that Beer's law holds for this solute in each solvent at the range of concentrations taken, was verified by experiment.)

*Reversed-phase Partition Chromatography of Resin Mixtures.*—Kieselguhr (Johns-Manville "Hyflo Super-Cel"), made water-repellent by treatment with dichlorodimethylsilane (Howard and Martin, 1950), was used to support the stationary phase. Among the hydrocarbons tried as stationary phase, *n*-octane, ligroin (b.p. 88–101°), and a $C_{12}$ hydrocarbon cut kindly supplied by Shell Chemicals, Ltd., were found useful; especially the last, which showed very little leakage of stationary phase when the mobile phase was flowing. The columns were packed by application of air pressure. The substances examined were often sparingly soluble in the mobile phase; it was then necessary to dissolve them in a little stationary phase, mix with kieselguhr, slurry with mobile phase, and apply to the top of the column. Aqueous methanol of various concentrations saturated with stationary phase was used as the mobile phase and was generally run under slight excess air pressure. Frac-

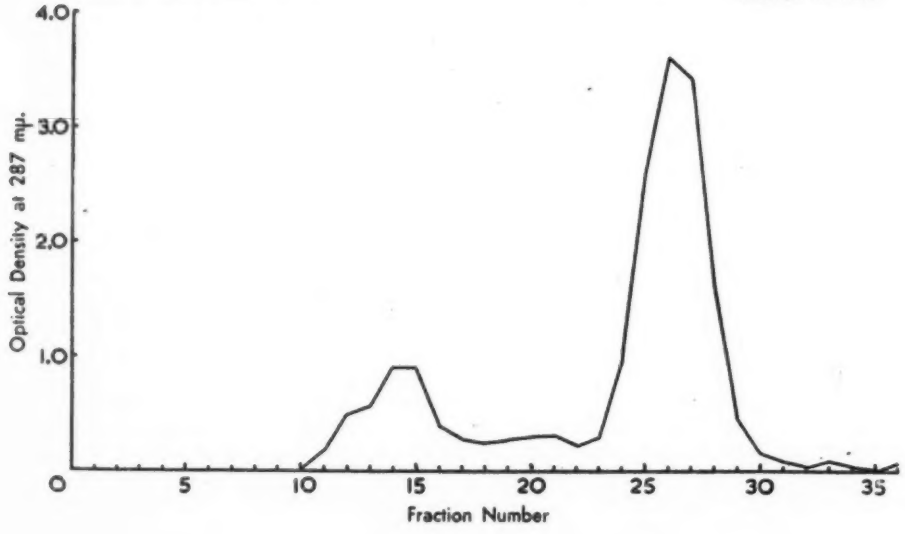



FIG. 7.—Partition chromatography of tetracyclic compound. Ordinate: optical density at 287 mμ. Abscissa: fraction number. ——— CCMD (8 mg.); silane-treated hyflo (12.5 g.); *n*-octane (3.75 ml.); 92.5% v/v aq. methanol. CCMD = contents of tube 5 in 2nd countercurrent distribution (Fig. 5) of twice molecularly distilled crude tetracyclic compound.

tions of 4 ml. volume were examined for absorption at 287 mμ.

A mixture of dicyclic and tricyclic phenols was not separated when 92.5% methanol was used, but 70% methanol gave good separation (Fig. 6). Analysis of the tetracyclic phenol (Fig. 7) showed that this was not free from lower homologues (dicyclic and perhaps tricyclic) even after two molecular distillations followed by two countercurrent distributions. The crude pentacyclic phenol (Fig. 8) was similarly shown to contain the tetracyclic phenol as well as lower homologues. Chromatography of $D_2$ resin gave a curve (Fig. 9) with successive peaks attributable to di-, tri-, tetra-, penta-, and hexacyclic phenols. The final peak in Fig. 9, obtained by substituting ethanol for methanol, is probably due to mixed higher homologues. A resin obtained by two successive precipitations of $D_2$ resin with methanol showed the expected lower proportion of dicyclic and tricyclic constituents (Fig. 9); a resin ($D_{25}$) obtained by repeated precipitation of $D_2$ resin with methanol showed no peak corresponding to polymers with less than seven phenolic nuclei.

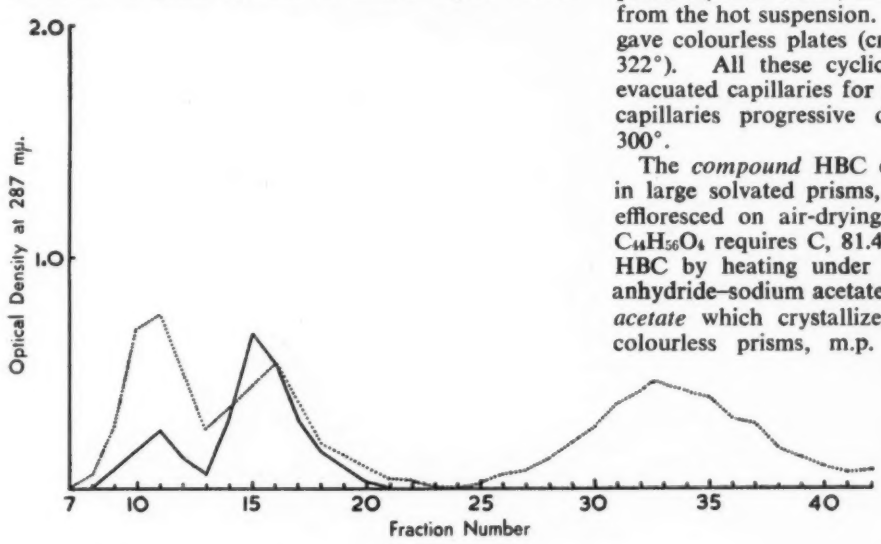



FIG. 8.—Partition chromatography of tetracyclic and pentacyclic compounds. Ordinate: optical density at 287 mμ. Abscissa: fraction number. ——— Crude tetracyclic compound (2½ mg.). ..... Crude pentacyclic compound (5 mg.) applied in stationary phase. Each column contained silane-treated hyflo (12.5 g.) and $C_{18}$ hydrocarbon (6.25 ml.). Mobile phase 96% v/v aq. methanol.

1:8:15:22 - *Tetrahydroxy* - 4:11:18:25 - *tetra* - tert. - *butyl* - cyclo - *tetra* - m - *benzylene* (VI; R = $C(CH_3)_3$).—The method of Zinke and Ziegler (1944) was modified, Dowtherm being used in place of linseed oil; *p-tert.*-butylphenol (10 g.) gave 3.2 g. crude product. This was boiled with acetic acid for half an hour; the insoluble residue was collected, dried, and boiled with toluene (30–50 ml.). The insoluble white powder (crude HBC; 1.6 g.; m.p. 380°) was collected from the hot suspension. Concentration of the filtrate gave colourless plates (crude LBC; 0.7 g.; m.p. 320–322°). All these cyclic compounds were sealed in evacuated capillaries for m.p. determination: in open capillaries progressive decomposition set in above 300°.

The *compound* HBC crystallized from chloroform in large solvated prisms, m.p. 380° (decomp.), which effloresced on air-drying (Found: C, 81.9; H, 8.8. $C_{44}H_{56}O_4$ requires C, 81.4; H, 8.7%). Acetylation of HBC by heating under reflux for 2 hr. with acetic anhydride–sodium acetate gave, on cooling, *HBC tetra-acetate* which crystallized from acetic anhydride in colourless prisms, m.p. 332–333° (decomp.). The substance was dried at 140° in high vacuum for analysis, but even so retained solvent of crystallization: when a toluene solution of the crystals was washed with water, the water became acidic (Found: C, 73.3, 73.2; H, 7.5, 7.8. $C_{52}H_{64}O_8$, $C_4H_6O_3$ requires C, 73.2; H, 7.6%). Another sample was analysed after crystallization from ethyl acetate-ligroin and drying

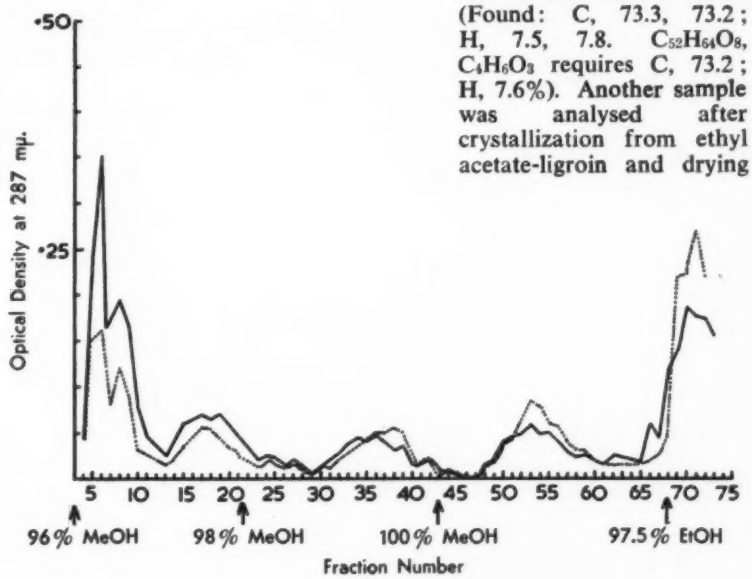



FIG. 9.—Partition chromatography of mixed resins. Ordinate: optical density at 287 mμ. Abscissa: fraction number. ——— $D_2$ resin (2 mg.). ..... Residue (3 mg.) from double methanol extraction of $D_2$ resin. Both chromatographed on silane-treated hyflo (6.25 g.) with $C_{18}$ hydrocarbon (3.13 ml.) as stationary phase.

at 140° (Found: C, 76.0; H, 8.1; M(Rast), 789, 832. $C_{52}H_{64}O_8$ requires C, 76.4; H, 7.9%; M, 816).

The *compound* LBC crystallized readily from chloroform in solvated prisms, m.p. 330–332° (decomp.), which still retained chloroform after prolonged heating *in vacuo*. Recrystallization from toluene gave plates, m.p. 320–321° (decomp.). These also contained solvent, but this was expelled on drying in high vacuum at 140° (Found: C, 82.7; H, 8.6; loss at 140°, 13.9%. $C_{44}H_{56}O_4,C_7H_8$ requires C, 82.7; H, 8.7; and contains 12.4% toluene. Found on dried material: C, 81.6; H, 8.7; M(Rast), 628, 623. $C_{44}H_{56}O_4$ requires C, 81.4; H, 8.7%; M, 648). Acetylation, as above, gave *LBC tetra-acetate* which crystallized from acetic acid in solvated prisms, m.p. 247–250° (Found: C, 73.7, 73.5; H, 7.9, 8.0; M(Rast), 879, 899. $C_{52}H_{64}O_8$, $C_2H_4O_2$ requires C, 73.9; H, 7.8%; M, 877). The apparent behaviour of this solute as a single molecule in the Rast determination is probably due to volatilization of acetic acid from the camphor solution.

Neither HBC nor LBC reacted with *p*-nitrobenzenediazonium chloride when dissolved in excess aqueous-alcoholic alkali. However, HBC did give a purple solution in this test when a minimum of alkali was used to dissolve the phenol. Both compounds failed to react with bromine in chloroform solution.

1:8:15:22 - *Tetrahydroxy* - 4:11:18:25 - *tetra* (1:1:3:3 - *tetramethylbutyl*) - cyclo*tetra* - m - *benzylene* (VI; R = $C(CH_3)_2.C(CH_3)_3$).—The following conditions are critical for maximum yields. Octylphenol (40 g.), 3N-NaOH (40 ml.) and aqueous formaldehyde (17 ml.; 33% w/v) were mixed in a jacketed tube and stirred occasionally for 1–2 hr., then heated at the boiling point of acetone for 6 hr. The product was shaken with toluene (80 ml.) and an excess of dilute HCl; the toluene layer was washed with saturated aqueous $NaHCO_3$. Sodium hydroxide (1.8 ml., 3N) was added to the toluene solution and the solvent was removed at lower pressure. The residue was slowly heated during 1–2 hr. at 0.5–1 mm. pressure to 210–220°, and this temperature was maintained for 1¼ hr. The product, a brittle opaque resin (34 g.), was powdered and suspended in boiling acetone (340 ml.). After a few minutes the white insoluble portion was collected and triturated with boiling acetone. The residue (crude HOC; 11–13 g.; m.p. > 300°) was a white powder.

The acetone filtrate was concentrated to half volume and benzene ( ~ 10 ml.) was added. After 1–3 days crude LOC (1.5–2 g.) was collected; m.p. 220–240°.

Crude HOC dissolved slowly in boiling benzene; after treatment with charcoal, acetone (2–3 vols.) was added and crystallization was allowed to proceed at 37°. The crystallized product was again triturated with boiling acetone and recrystallized first from benzene and finally from benzene-acetone at 37°. The *compound* crystallized in rods, m.p. 315° up to 335° (decomp.), depending on the rate of heating (Found: C, 82.6, 82.5; H, 10.3, 9.8. $C_{60}H_{88}O_4$ requires C, 82.5; H, 10.2%). Acetylation gave *HOC tetra-acetate* which crystallized from acetic anhydride in colourless prisms, m.p. 274.5–275.5° (Found: C, 78.7; H, 8.9; M(Rast), 723. 810. $C_{68}H_{96}O_8$ requires C, 78.4; H, 9.3%; M, 1040). The *tetra-β-hydroxyethyl ether* of HOC was obtained by heating a solution of HOC (2 g.) in toluene (28 ml.) with ethylene oxide (7.9 ml.) and diethylamine (0.15 ml.) in an autoclave at 140–150° for 3½ hr. Slow evaporation of the toluene gave colourless plates (1.4 g.; m.p. 280–285°) which were purified by four recrystallizations from ethyl acetate and then had m.p. 292–293° (Found: C, 77.8; H. 10.1; M(Rast), 1030, 1340. $C_{68}H_{104}O_8$ requires C, 77.8; H, 10.0%; M, 1049).

Crude LOC was purified by two recrystallizations from toluene (charcoal); the *compound* had m.p. 246° (Found: C, 82.9, 82.7; H, 9.9, 9.8. $C_{60}H_{88}O_4$ requires C, 82.5; H, 10.2%). The *tetra-acetate* crystallized from acetic acid in colourless rods, m.p. 259–260° (Found: C, 78.5, 78.5; H, 9.9, 9.4; M(Rast), 826, 874. $C_{68}H_{96}O_8$ requires C, 78.4; H, 9.3%; M, 1040).

*Reaction of Phenols with Ethylene Oxide.*—A stainless steel autoclave of 100 ml. capacity, fitted with a pressure gauge, was used in all the following experiments. In general, the phenolic substance (0.5–2.0 g.) in toluene was placed in the autoclave and powdered NaOH was added. The autoclave was cooled to 0° and sealed after introduction of liquid ethylene oxide; it was then (sometimes at once, sometimes next day) immersed vertically in an oil-bath at 140°. This

TABLE I
CRYSTALLOGRAPHIC DATA

| | a | b | c | β | ρ | Space Group | n | M Asymmetric Unit | M Calc. for Tetrapolymer |
|---|---|---|---|---|---|---|---|---|---|
| HOC .. | 23·42 | 20·68 | 46·5 | 102° | 1·074 | $P2/c$ | 4 | 3,555 | 876 |
| HOC acetate | a sin γ = 15·2 | 13·6 | $d_{001}$ 14·7 | | 1·055 | P1 or $P\bar{1}$ | 1 or 2 | 1,910 or 955 | 1,048 |
| HBC .. | 20·1 | 16·9 | $d_{001}$ 18·1 | | 1·04 | P1 or $P\bar{1}$ | 1 or 2 | 3,920 or 1,960 | 652 |
| LOC .. | 11·38 | 38·5 | 12·58 | 100° | 1·068 | $P2_1/c$ | 4 | 884 | 876 |
| LBC .. | 12·72 | 12·72 | 14·0 | | 1·09 | P4/n | 8 | 648/4 (Corr. for solvent) | 648 |

temperature was maintained until no further fall in pressure was perceptible, when the autoclave was cooled and opened. The toluene solution was steam-distilled until frothing became copious; the aqueous solution was roughly neutralized, transferred to a large, weighed flask and evaporated on a steam-bath at low pressure. Water was then added, and the *p*H adjusted, to give a 25% w/w solution at *p*H 7, which was diluted for pharmacological and chemotherapeutic tests with an equal volume of physiological saline. Aliquots of the toluene or water solutions were sometimes evaporated to check whether reaction was complete; the weight of product so determined was always within a few per cent of the theoretical.

TABLE II

ETHYLENE OXIDE CONDENSATION PRODUCTS

Generally, 2 g. of the appropriate phenol, 10 ml. toluene, 0.15 g. NaOH, and the appropriate volume of ethylene oxide were heated at 140° until reaction was complete (usually 1–2 hr.)

| Series and Code No. | Chemical Formula |
|---|---|
| Tr-30 ($D_{13}$) | II; $n=1$; $x=30$ |
| $D_2$–10·5 ($D_{14}$) | II; $n=0, 1, 2, 3$, etc.; $x=10·5$ |
| M–10 ($D_{15}$) | IV; $x=10$ |
| Tr–10 ($D_{16}$) | II; $n=1$; $x=10$ |
| Di–10 ($D_{17}$) | II; $n=0$; $x=10$ |
| Di–29 ($D_{18}$) | II; $n=0$; $x=29$ |
| M–30 ($D_{19}$) | IV; $x=30$ |
| M–60 ($D_{21}$) | IV; $x=60$ |
| $D_2$–60 ($D_{22}$) | II; $n=0, 1, 2, 3$, etc.; $x=60$ |
| $D_2H$–20 ($D_{25}$) | II; $n=5, 6, 7$, etc.; $x=20$ |

In Table II the ethylene oxide condensation products from compounds of general formula (II; R = H) are listed along with some, prepared from octylphenol, which have the formula (IV). These are compounds not reported in our previous communication.

In Tables III and IV are reported, in somewhat greater detail, the preparation of several products—chiefly from the macrocyclic phenols—which are referred to by code number in the following section.

## PHARMACOLOGICAL SECTION

### MATERIALS AND METHODS

*Surface-active Agents.*—For biological testing the agents were made up as 12.5% (w/v) solutions in 0.45% (w/v) saline, adjusted to *p*H 7 and autoclaved. They were usually clear yellow fluids, frothing readily on shaking. The viscosity of the solution varied inversely with the average ethylene oxide chain length of the products; it was marked only in those compounds with less than 15 units of ethylene oxide. For simplicity the surface-active agents will be referred to by series and code number only, but all are described in detail in the earlier part of this paper. They consist of eight series of polyoxyethylene ethers, derived from the following eight intermediates: M (monocyclic-*p-tert.*-octylphenol), Di (linear dicyclic *p-tert.*-octylphenol formaldehyde compound), Tr (linear tricyclic *p-tert.*-octylphenol formaldehyde compound), $D_2$ (linear polycyclic *p-tert.*-octylphenol formaldehyde mixture; $D_2H$ is a derivative of $D_2$ but is free from compounds with fewer than 7 phenolic nuclei), LBC and HBC (a pair of stereoisomers of a macrocyclic tetra-nuclear *p-tert.*-butylphenol formaldehyde compound), and LOC and HOC (a pair of stereoisomers of a macrocyclic tetra-nuclear *p-tert.*-octylphenol formaldehyde compound). The final products are referred to their parent intermediate by indicating the average number of molecules of ethylene oxide per chain (HOC-20, etc.).

*Tuberculostatic Tests.*—The agents were tested against the human virulent strain of *Mycobact. tuberculosis*, H37Rv, in a defined liquid medium containing 0.25% (w/v) albumin (Hart and Rees, 1954).

*Chemotherapeutic Tests.*—Female mice (18–20 g.) of the albino P strain were infected intravenously by the injection of 0.1 mg. (wet-weight bacilli) of the strain H37Rv, prepared by grinding a fully-grown culture obtained from the surface of Proskauer and Beck's medium. This method produced an acute tuberculous infection, resulting usually in the death within 30 days of all the untreated control mice with

TABLE III

PREPARATION AND CODE NO. OF COMPOUNDS

| Series and Code No. | Wt. of Phenol (g.) | Vol. of Toluene (ml.) | Wt. of Catalyst (NaOH) (mg.) | Av. No. Ethylene Oxide Residues/ Phenolic Group | Time at Room Temp. before Heating (Hr.) | Bath Temp. Range (°C.) | Total Time of Heating (Hr.) |
|---|---|---|---|---|---|---|---|
| $D_2$–20 (GN/2) | 2·00 $D_2$ resin[1] | 8 | 100 | 20 | <3 | 140–150 | 1·3 |
| $D_2$–27½ (JF/1) | 2·00 $D_2$ ,, [1] | 28 | 100 | 27·5 | 16 | 140–150 | 0·9 |
| $D_2$–60 (GN/87) | 2·00 $D_2$ ,, [1] | 13 | 85 | 60 | <1 | 140–160 | 2·6 |
| $D_2$–60 (GN/112) | 2·00 $D_2$ ,, [1] | 13 | 105 | 60 | <1 | 135–145 | 2·0 |
| $D_2$–90 (GN/99) | 1·00 $D_2$ ,, [1] | 12 | 60 | 90 | <1 | 135–150 | 2·7 |
| LOC–45 (GN/93) | 2·00 LOC | 25 | 102 | 45 | 16 | 140–150 | 3·0 |
| LOC–60 (GN/90) | 2·00 LOC | 25 | 100 | 60 | 16 | 140–150 | 2·0 |
| LOC–75 (GN/92) | 2·00 LOC | 25 | 103 | 75 | 16 | 140–150 | 2·7 |
| HOC–45 (GN/103) | 2·00 HOC | 25 | 115 | 45 | <1 | 140–150 | 2·2 |
| HOC–60 (GN/102) | 2·00 HOC | 25 | 80 | 60 | <2 | 140–150 | 2·7 |
| HOC–75 (GN/104) | 1·00 HOC | 13 | 75 | 75 | <1 | 140–155 | 2·9 |

[1] These five samples were all from the same batch of resin.

TABLE IV

PREPARATION AND CODE NO. OF COMPOUNDS

| Series and Code No. | Wt. of Phenol (g.) | Solvent Vol. (ml.) a=toluene b=benzene | Wt. of Catalyst (NaOH) (mg.) | Av. No. Ethylene Oxide Residues/ Phenolic Group | Time at Room Temp. before Heating (Hr.) | Bath Temp. Range (°C.) | Total Time of Heating (Hr.) |
|---|---|---|---|---|---|---|---|
| LBC–15 (GN/14) .. .. | 0·50 | 20a | 29 | 15 | ?16 | 135–145 | 5·5 |
| HBC–15 (GN/72) .. .. | 0·50 | 25b | 40 | 15 | <1 | 135–145 | 5·0 |
| HBC–20 (GN/71) .. .. | 0·50 | 25b | 36 | 20 | 48 | 135–145 | 4·5 |
| LOC–15 (GN/76) .. .. | 1·00 | 16a | 60 | 15 | 16 | 140–160 | 8·5 |
| LOC–20 (GN/73) .. .. | 1·00 | 16a | 64 | 20 | 16 | 140–150 | 3·75 |
| LOC–25 (GN/82) .. .. | 2·00 | 33a | 92[1] | 25 | 16 | 135–145 (from r.t.) | 2·75 |
| LOC–30 (GN/81) .. .. | 2·00 | 32a | 100 | 30 | 16 | 135–145 (from r.t.) | 2·25 |
| HOC–20 (GN/30) .. .. | 0·50 | 7a | 14, 27, 40[2] | 20 | <1 | 140–150 | 3·3, 2·1, 1·6[3] |
| HOC–20 (GN/57) .. .. | 0·50 | 7a | 26 | 20 | <1 | 140–150 | 4, 10[3] |
| HOC–15 (GN/79) .. .. | 2·00 | 28a | 95 | 15 | <1–3 | 140–165 | 8[4] |
| HOC–17 (GN/80) .. .. | 2·00 | 28a | 75 | 17 | 3 | 140–150 | 4 |
| HOC–20 (GN/68) .. .. | 2·00 | 28a | 116 | 20 | <1 | 140–150 | 3 |
| HOC–25 (GN/70) .. .. | 1·00 | 14a | 50 | 25 | <1 | 130–150 | 4 |

[1] Catalyst and solution heated under reflux before transfer to bomb. [2] At two stages the bomb was left overnight and more catalyst added. [3] Heating in two stages. [4] Bomb left at room temperature overnight after heating for first 2 hr.

gross pulmonary tuberculosis (average survival time 21 days). A standard therapeutic procedure was used, the treated mice (10 or 12/group) being given three intravenous injections of the agent at 25 mg., or at the maximum tolerated dose if less, contained in 0.2 ml. saline, on the 1st, 4th, and 8th days after infection (technical difficulties sometimes made it impossible to give the 2nd or 3rd i.v. dose, or both, and these failures were recorded by marking the mice). The experiments were terminated on the 34th day after infection. The amount of gross tuberculosis of the lung, heart, and kidney was recorded at autopsy. Chemotherapeutic activity of the agent was assessed from the median survival times, and from the amount of macroscopic pulmonary tuberculosis in any animal surviving to the 34th day, in the treated and control groups.

*Pharmacological Tests.*—Female mice of the albino P strain, 18–20 g. initial weight, were used.

(*a*) *Toxicity.* The approximate acute LD50 of each agent was determined 24 hr. after a single dose administered intravenously. Chronic toxicities were determined (for some of the agents only) by daily subcutaneous doses, or twice weekly intravenous doses, given over a period of 2 weeks.

(*b*) *Lipaemia Production.* Blood was taken from the hearts of mice surviving to the end of the 24-hr. acute toxicity tests. The blood was allowed to clot, centrifuged, and the serum examined with the naked eye for milkiness.

(*c*) *Adrenal Cortical Changes.* The same mice surviving 24 hr. in the acute toxicity tests were killed, and the adrenal glands examined with the naked eye for colour changes in the cortex. It has been shown previously (Rees, 1953), using histochemical tests, that a clear pink adrenal cortex (instead of the normal yellow colour) has no detectable lipid.

## RESULTS

In vitro *Activity.*—With the exception of M-10 ($D_{15}$) and M-30 ($D_{19}$) none of the present agents, where tested, inhibited the growth of tubercle bacilli at the highest concentrations used (0.2–2.0%, see Table V). Their ability to promote dispersed growth was recorded (see Table V) during these *in vitro* tuberculostatic tests. Some produced dispersed growth, comparable to that obtained with "Triton WR1339," whereas the others had little or no effect, and none was as good a dispersing agent as "Tween 80." There was no correspondence between ability to disperse and chemotherapeutic activity.

*Pharmacological Action.*—From the results (Table V) it can be seen that, with the exception of the agents of smaller molecular weight (M-10, M-60, Di-10, and Tr-10), these compounds could be given as single intravenous injections to mice in large doses without causing death. On the other hand, many of them had a profound effect on the body lipids, as shown by the production of gross lipaemia and the removal of lipids from the adrenal cortex. These changes, 24 hr. after a single intravenous injection of the agent, are shown in the Table. Although blood cholesterol estimations were not made, an earlier study with somewhat similar products (Cornforth *et al.*, 1951) showed that gross lipaemia was regularly associated with at least a twofold increase in blood cholesterol concentration. More definitive experiments designed to study the production of lipaemia by the present agents have shown that lipaemia remains for at least 36 hr., although it appears

within 30 min. ; it also occurs in adrenalectomized mice. The absence of lipaemia production and of stripping of the adrenal cortical lipids by some of the members of the HOC series (e.g., HOC-20) was in sharp contrast to the effect of members of the $D_2$ and LOC series having similar average ethylene oxide chain lengths. HOC-20 (GN/57) was therefore subjected to a more sensitive test, using the rabbit and giving three intravenous injections (312 mg./kg.) of the agent on alternate days. Blood samples taken 48 hr. after the last injection showed no lipaemia and no rise in blood cholesterol. By contrast, both $D_2$-20 and the commercial product ("Triton WR1339"), at these doses, produced in rabbits marked lipaemia with the usual associated rise in cholesterol concentration.

Some of the products (including $D_2$-10.5, $D_2$-20, $D_2$-60, and HOC-20) were subjected to chronic toxicity tests in mice. Although in every test the animals gained weight and seemed healthy, microscopical examination revealed liver damage. Considerable individual variation was observed, for, although in more than half there were only a few focal areas of necrosis, the others showed much more extensive liver damage.

*Chemotherapeutic Activity.*—Table V shows variable activity: an anti-tuberculous effect is

TABLE V

PHARMACOLOGICAL DATA

| Surface-active Agent Series and Code No. | Inhibitory Concn. (g./100 ml.)[1] and Type of Growth at Sub-inhibitory Concn.[2] | | Acute i.v. Toxicity LD50 in mg. (Single Injection) | Blood and Adrenal Cortex Changes (24 Hr. after Single i.v. Injection) | | Chemotherapeutic Activity[5] (Dose, mg.) |
|---|---|---|---|---|---|---|
| | | | | Lipaemia[3] (Dose, mg.) | Loss of Adrenal[4] Cortical Lipid (Dose, mg.) | |
| M–10 ($D_{16}$) | 0·012 | g | 2·0‡ | 0 (2) | 0 (2) | — |
| M–30 ($D_{19}$) | 0·05 | g | 7·5 | 0 (7·5) | 0 (7·5) | — |
| M–60 ($D_{21}$) | >0·5 | g | 25·0 | 0 (25) | 0 (25) | 0 (15) |
| Di–10 ($D_{17}$) | >0·5 | d | 5·0‡ | 0 (5) | 0 (5) | — |
| Di–29 ($D_{18}$) | >0·5 | d | 15·0 | 0 (15) | 0 (15) | 0 (10) |
| Tr–10 ($D_{15}$) | >2·0 | d | 5·0‡ | 0 (5) | 0 (5) | — |
| Tr–30 ($D_{13}$) | >2·0 | d | >25·0 | + (25) | + (25) | 0 (15) |
| $D_2$–10·5 ($D_{14}$) | >2·0 | d | >12·5 | + (12·5) | + (12·5) | +++ (12·5) |
| $D_2$–20 (GN/2)* | >2·0 | d | >50·0 | + (15–50) | + (15–50) | +++ (25) |
| $D_2$H–20 ($D_{25}$) | — | — | >50·0 | + (25–50) | + (25–50) | ++ (25) |
| $D_2$–27·5 (JF/1) | — | — | >50·0 | + (50) | + (50) | 0 (25) |
| $D_2$–60 (GN/87) | — | — | >50·0 | 0 (50) | 0 (50) | 0† (25–65) |
| $D_2$–60 ($D_{23}$) | >2·0 | g | >65·0 | 0 (25) | 0 (25) | 0† (25) |
| $D_2$–90 (GN/99) | — | — | >50·0 | 0 (50) | 0 (50) | 0† (25) |
| LBC–15 (GN/14) | >2·0 | d | 12·5 | + (10) | + (10) | 0 (10) |
| HBC–15 (GN/72) | >2·0 | d | >50·0 | + (15–50) | + (25–50) | ++ (25) |
| HBC–20 (GN/71) | — | — | >50·0 | + (25–50) | + (50) | + (25) |
| LOC–15 (GN/76) | — | — | >50·0 | + (10–50) | + (20–50) | +++ (25) |
| LOC–20 (GN/73) | >2·0 | d | >50·0 | + (10–50) | + (25–50) | +++ (25) |
| LOC–25 (GN/82) | — | — | >50·0 | + (10–50) | + (10–50) | 0 (25) |
| LOC–30 (GN/81) | >2·0 | d | >50·0 | + (10–50) | + (20–50) | 0 (25) |
| LOC–45 (GN/93) | — | — | >50·0 | + (10–50) | + (15–50) | 0† (25) |
| LOC–60 (GN/90) | >0·2 | d | >50·0 | + (10–50) | + (15–50) | 0† (25) |
| LOC–75 (GN/92) | — | — | >50·0 | + (10–50) | + (25–50) | 0† (25) |
| HOC–15 (GN/79) | >2·0 | g | >50·0 | + (50) | + (50) | +++ (25) |
| HOC–17 (GN/80) | — | — | >50·0 | + (50) | + (50) | ++ (25) |
| HOC–20 (GN/68) | — | — | >50·0 | 0 (50) | 0 (50) | + (25) |
| HOC–20 (GN/30) | — | — | >50·0 | 0 (50) | 0 (50) | +++ (25) |
| HOC–20 (GN/57) | >0·2 | g | >25·0 | 0 (50) | 0 (50) | +++ (25) |
| HOC–25 (GN/70) | >2·0 | g | >50·0 | 0 (50) | 0 (50) | 0 (25) |
| HOC–45 (GN/103) | — | — | >50·0 | 0 (50) | 0 (50) | 0† (25) |
| HOC–60 (GN/102) | >0·2 | g | >50·0 | 0 (50) | 0 (50) | 0† (25) |
| HOC–75 (GN/104) | — | — | >50·0 | 0 (50) | 0 (50) | 0† (25) |

* This surface-active agent was referred to as $D_2$ in our earlier papers (Cornforth *et al.*, 1951; Rees, 1953; Hart *et al.*, 1952).

[1] No growth at 2 weeks of *Mycobact. tuberculosis* (H37Rv).

[2] g, granular or partly dispersed growth; d, dispersed growth, comparable to that obtained with "Triton WR1339."

[3] 0: no lipaemia; +: lipaemia.

[4] 0: no loss of adrenal cortical lipoids; +: loss of adrenal cortical lipoids.

[5] 0: inactive; +: low activity, i.e., significant but slight prolongation of median survival time but most of treated mice died of tuberculosis by 34th day. ++: moderately high activity, i.e., considerable prolongation of survival time but more than half treated mice died of tuberculosis by 34th day. +++: high activity, i.e., not more than one treated mouse died of tuberculosis by the 34th day, little macroscopic pulmonary tuberculosis in the surviving mice killed on the 34th day. 0†: "protuberculous," i.e., no chemotherapeutic activity, treated mice dying significantly earlier and with more fulminating tuberculosis than the untreated controls.

‡ Intravascular haemolysis.

confined to the $D_2$, HBC, LOC, and HOC series and only to those members with an average chain-length of 20 or less ethylene oxide units. The highest activity encountered (+++, see Table V) is comparable with that obtained using streptomycin (2 mg. daily) in a similar mouse test. By analysing the protection produced in those mice that received no more than the first intravenous dose of the more active products, it was evident that a single injection given on the first day after infection was as therapeutically effective as the full course.

During this work it was noted that, although some products were inactive (0, see Table), others caused a significant enhancement of the tuberculous infection as compared with the control group. The products showing this effect, which include all members of the $D_2$, LOC, and HOC series with polyoxyethylene chain-lengths of 45 or more units, are recorded here as "Protuberculous" (0†, see Table).

It has been shown previously (Hart, Long, and Rees, 1952) that a number of our earlier chemotherapeutically active products ($D_2$-10.5 and $D_2$-20) depress tuberculin sensitivity in BCG-vaccinated guinea-pigs. Although our newer series were not examined systematically for this property, two therapeutically active representatives (HOC-20 (GN/57) and LOC-20 (GN/73)) were found to possess it.

## Discussion

The results now obtained with these eight series of surface-active polyoxyethylene ethers make it possible to define more precisely the structural requirements for antituberculous activity. It is also possible, for the first time, to exclude some of the associated biological properties of these agents as necessary for chemotherapeutic activity.

We can now be sure that the suppression of experimental tuberculosis by the commercial detergent "Triton A20," first reported, was due to the main constituent and not to some unknown chemical contaminant. Since then, this biological property has been found in a variety of allied phenolic condensation products. Some of our newer surface-active agents are derived from phenolic condensation products having a macrocyclic instead of a linear structure. Moreover, the intermediates are pure and crystalline (almost certainly tetracyclic) rather than a mixture, as in the $D_2$ series. Four such series have been made, two from macrocyclic *p-tert.*-butylphenol (LBC and HBC) and two from macrocyclic *p-tert.*-octylphenol (LOC and HOC) intermediates. Some of the macrocyclic products (HBC-, LOC-, and HOC-) are as active as the linear, and the *p-tert.*-butyl grouping is little less effective than *p-tert.*-octyl.

The previous suggestion (Cornforth *et al.*, 1951) that products derived from the smaller molecular size linear phenolic intermediates (1–3 phenolic nuclei, i.e., M, Di, and Tr series) have no therapeutic activity is confirmed. Activity is not achieved in the linear polynuclear series ($D_2$) till mixtures containing products with more than three phenolic nuclei is reached; the fact that $D_2$H-20 (mixture of condensation products with 7 or more phenolic nuclei) has the same order of activity as $D_2$-20, extends still further the range of active products. It is of interest that the M, Di, and Tr series have a high toxicity in mice and produce considerable haemolysis *in vitro* and *in vivo*. These acute toxic properties decrease with increased molecular size, even with these three series, and are absent from the still larger molecular-sized products from the other five series.

So far we have discussed alterations in the alkylphenol constituent of the molecule in relation to chemotherapeutic activity, but we became aware, from study of the linear series, that the lengths of the ethylene oxide chains were also important. Introduction of the macrocyclic series (HOC and LOC) has defined this factor more precisely. The great activity obtained in these two series by members having an average chain of 15-20* ethylene oxide units was abolished by a small increase to an average chain of 25–30 ethylene oxide units. Moreover, when a chain of 45–75 ethylene oxide units was present, a new biological phenomenon was noted. Although the resulting products showed no chemotherapeutic activity against experimental tuberculosis they were far from inert, for they significantly enhanced the infection (so-called "protuberculous effect"). This unexpected, but consistent, result has been examined in greater detail and will be the subject of a separate communication. It can be concluded that in these three homologous series ($D_2$, HOC, and LOC) alterations in the lipophilic-hydrophilic balance of the individual products (produced by changing the ethylene oxide chain-length) can profoundly modify the outcome of tuberculous infection. As the lipophilic/hydrophilic ratio decreases, activity passes from antituberculous→inactive→"protuberculous."

* In the HOC-20 series, variations in chemotherapeutic activity have been observed with different batches, a higher activity being associated with smaller scale runs. Investigations by Dr. P. C. Spensley suggest that this is due to a different statistical distribution of ethylene oxide chain-lengths and to somewhat less complete reaction with ethylene oxide in the smaller runs.

The commercial product "Triton WR1339" and our earlier series of linear surface-active agents produced lipaemia, associated with an increase of blood cholesterol and stripping of lipids from the adrenal cortex. At one time it was considered that this phenomenon might be related to chemotherapeutic activity. The high therapeutic activity of some of the HOC series, which produce none of these disturbances in lipid metabolism, now makes it quite clear that these associated effects are not essential. This conclusion confirms our previous (unpublished) observations that: (*a*) smaller doses of $D_2$-20, insufficient to produce lipaemia and stripping of adrenal lipids, though still raising the level of blood cholesterol, were therapeutically effective; (*b*) a high level of blood cholesterol, maintained by feeding a cholesterol/cholate mixture to mice, did not protect against the standard tuberculous infection.

These products do not appear to be directly antibacterial, for none of those therapeutically active inhibits the growth of tubercle bacilli, even at high concentrations, *in vitro*. Neither has it been possible to detect a directly tuberculostatic substance in the blood or tissue fluids of treated animals. We have thus been led to consider that these agents may act primarily through the host. The finding that some products enhance, while others suppress, a tuberculous infection, in itself suggests an effect mediated through the host rather than directly on the tubercle bacillus.

A number of unsuccessful attempts to find such a mechanism have been reported previously (Rees, 1953). One of the most striking effects produced by some of these agents on the host—altered fat metabolism—can now also be excluded as an operative factor in chemotherapy (see above). More recently, Mackaness (1954) has shown that monocytes harvested from animals previously treated with the commercial agent ("Triton WR1339") become altered in some way so that tubercle bacilli that have been ingested by them are partially or completely inhibited in their growth, or even destroyed. The way in which these agents enhance *in vivo* the ability of monocytes to suppress the multiplication of intracellular tubercle bacilli is at present unknown, but these observations strongly suggest the monocyte as a site of action. This suggestion is further strengthened by using a dye readily solubilized by this type of surface-active agent to demonstrate that the agents do enter the monocyte *in vivo* (Lovelock and Rees, 1955).

One property of surface-active substances is to facilitate dispersed growth of tubercle bacilli, but dispersion in the test-tube is not correlated with activity against tuberculosis in the body, since inactive as well as active members of our series produce dispersed growth. Nevertheless, it seems probable that chemotherapeutic activity of these agents is related, in some way, to their surface-active properties. If this idea is confirmed then a new type of antituberculous agent, based on a physical rather than a chemical action, will have been established.

## Summary

1. The preparation is described of linear condensation products from *p*-octylphenol and formaldehyde, including homogeneous dicyclic and tricyclic compounds and less pure tetracyclic and pentacyclic compounds.

2. A method of separating mixtures of linear condensation products is described.

3. Two pairs of crystalline macrocyclic condensation products were prepared from *p-tert.*-butylphenol and from *p-tert.*-octylphenol with formaldehyde. These were shown to be, almost certainly, stereoisomeric forms of a macrocyclic structure with four phenolic nuclei.

4. Conditions are described for the condensation of all these products with ethylene oxide.

5. The effect on tuberculous infection and some of the pharmacological properties of the condensation products are given. The products are arranged in eight series.

6. Some members of the series show high chemotherapeutic activity against a standard tuberculous infection in mice, comparable with that obtained using streptomycin.

7. In determining therapeutic activity ethylene oxide chain-length is critical. Maximum activity is obtained with products having an average ethylene oxide chain-length of 15–20 units.

8. As the ethylene oxide chain-length is increased the effect on tuberculous infection changes from antituberculous→inactive→"protuberculous" (average length of 45 units or more).

9. None of the chemotherapeutically active members of the series inhibits the growth of tubercle bacilli, even at high concentrations, *in vitro*. No tuberculostatic substance has been detected in the blood or tissue fluids obtained from treated animals.

10. It is suggested that these agents act indirectly through the host rather than by direct antibacterial action *in vivo*.

11. The possible site and mode of action of these agents is discussed.

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# HISTAMINE RELEASE IN RABBIT BLOOD BY DEXTRAN AND DEXTRAN SULPHATE

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Injection of dextran into rats, either intravenously or intraperitoneally, gives rise to reactions having certain similarities to allergic manifestations in man (Morrison, Bloom and Richardson, 1951a). Such reactions are characterized by marked oedema of the feet, jowls and nose, stupor and dyspnoea, followed by scratching (Voorhees, Baker and Pulaski, 1951; Briot and Halpern, 1952a). Urticarial reactions in man following intravenous administration have also been reported (Wilkinson and Storey, 1953). Briot and Halpern (1952b) showed that promethazine and adrenaline both gave some protection against the effects of injected dextran in rats; but Morrison, Richardson and Bloom (1951b), using a variety of antihistamine agents, found only phenindamine ("Thephorin") to be effective. Protection was also obtained by pretreatment with cortisone (Swingle, Fedor, Maxwell, Ben, and Barlow, 1953; Swingle, Fedor, Ben, Maxwell, and Baker, 1953).

Anaphylactoid type reactions in guinea-pigs have been reported following the parenteral administration of certain samples of dextran sulphate with molecular weights above a certain critical level (Walton and Ricketts, 1954). These reactions were not prevented by antihistamine drugs.

In order to determine whether reactions due to dextran or dextran sulphate could be attributed in part to the effects of released histamine, it seemed of interest to determine the effect of incubating them with rabbit blood, the cellular histamine of which is known to be released by a variety of agents capable of causing anaphylactic or anaphylactoid reactions *in vivo* (Code, 1952).

## METHODS

*Histamine Release in Blood.*—All glass apparatus used to handle blood was treated with Silicone D.C. 1107. Pipettes were again treated after they had been used three or four times, but centrifuge tubes in which histamine release had taken place were immersed in a sulphuric acid-chromate cleaning mixture overnight, washed, and then re-treated with silicone before being used again. Rabbit blood was obtained from the incised marginal vein of the ear after the hair surrounding the vein had been clipped short. Those parts of the ear likely to come into contact with blood were carefully smeared with soft paraffin, and the skin at some distance from the incision was gently rubbed with a benzene-impregnated swab to ensure a good flow of blood. Blood was collected in a tube containing ice-cold heparinized saline (NaCl 0.9% w/v, Heparin Evans 3 units/ml.) supported in a beaker containing crushed ice to minimize non-specific histamine release from the cells.

Histamine release reactions were carried out in 50 ml. round-bottomed centrifuge tubes at 37.5° C. The required number of tubes containing equal volumes of the same sample of dilute heparinized blood (usually 5 or 6 ml.), and also those containing solutions of the histamine-releasing agents or inhibitors, were placed in a water bath at 37.5° C. and were left for 15 min. for their contents to reach bath temperature. Histamine-releasing agents or inhibitors were dissolved in 0.9% w/v saline. The volume of such solution added to a tube of dilute blood was always 1 ml., and mixing was accomplished by rapidly swirling the tube. Inhibitors, when used, were always added to blood immediately before the histamine-releasing agents. A control sample of untreated dilute blood was included in each experiment, and equal volumes were maintained in all tubes at each stage by the addition of saline at 37.5° C.

Histamine release was allowed to take place for 30 min. The blood samples were then chilled in ice and centrifuged together at 3,000 rev./min. for 20 min. at room temperature. An aliquot of the supernatant dilute plasma was removed from each tube immediately and the histamine extracted.

*Extraction and Assay of Histamine.*—4.5 ml. samples of dilute plasma were submitted to the extraction procedure described by McIntire, Roth, and Shaw (1947) within a few minutes of removal from the cells. The final acid eluates were adjusted to *p*H 7.0 with indicator papers reading to 0.3 of a unit and made up to volume just before assay. Histamine present in the eluates was assayed biologically on guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. Most histamine estimations were carried out with an automatic organ-bath apparatus (Boura, Mongar, and Schild, 1954; Schild,

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1947) by the 2×2 assay technique (Schild, 1942) using 5 groups of 4 responses, though a few solutions were assayed in a hand-operated organ bath by bracketing against the responses from a standard histamine solution.

Concentrations of plasma histamine were calculated as μg. of base per ml. of blood, disregarding the volume occupied by the cells.

As a check on the reliability of the techniques used, two samples of rabbit plasma were extracted and assayed for their histamine content several times. The mean estimate of histamine present in the first was 0.22 μg./ml., S.E. 0.02 μg. (7 extractions and assays), and in the second 0.35 μg./ml., S.E. 0.025 μg. (5 extractions and assays). Recovery experiments were also carried out by estimating the histamine content of samples of rabbit plasma with and without added histamine. In 11 determinations the histamine found, calculated as a percentage of the expected value, was 90.0% (S.E. 3%).

Details of the polysaccharides used in these experiments were supplied by Dr. C. R. Ricketts and are given in Table I. Most of the data and the methods used to determine the intrinsic viscosities and molecular weights of the compounds have been published (Ricketts, 1952a, 1952b; Ricketts and Walton, 1952; Walton, 1952).

TABLE I

SUMMARY OF THE POLYSACCHARIDES TESTED EITHER AS HISTAMINE RELEASING AGENTS OR AS INHIBITORS OF HISTAMINE RELEASE

| Polysaccharide | Anticoagulant Activity (Units/mg.)* | Approximate Molecular Weight | Intrinsic Viscosity of Parent Dextran |
|---|---|---|---|
| Dextran I .. .. | | <14,000 | 0·02 |
| ,, E .. | | 22,000 | 0·12 |
| ,, .. .. | | 80,000 | 0·25 |
| ,, D .. .. | | 220,000 | 0·36 |
| ,, .. .. | | 1,000,000 | 0·64 |
| Heparin, Evans .. | 93 | | |
| Maltotriose sulphate | 10 (approx.) | | |
| Dextran sulphate I | 15–20 | <10,000 | 0·02 |
| ,, ,, E/1 | 15 | 40,000 | 0·12 |
| ,, ,, D/3 | 15 | 440,000 | 0·36 |

* In terms of International Standard Heparin, 130 units/mg.

## RESULTS

### *Histamine Release by Dextran and Dextran Sulphate*

Preliminary experiments showed that incubation of dilute heparinized rabbit blood with dextran sulphate D/3 led to a rise in the level of plasma histamine. Histamine release was initiated by quantities in the region of 0.1 μg./ml. It appeared that 4 μg./ml. was sufficient to release the greater part of the available cellular histamine, since, when the concentration was raised still further to 100 μg./ml., only slight increases in the levels of plasma histamine were obtained.

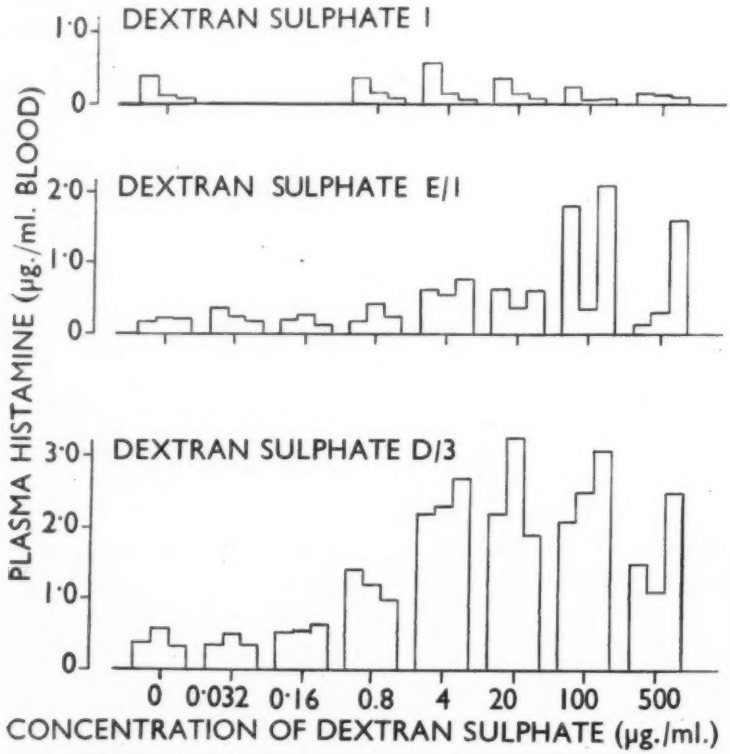


FIG. 1.—Release of histamine from rabbit blood cells by dextran sulphate. Plasma histamine levels after incubating dilute heparinized rabbit blood with varying concentrations of dextran sulphate for 30 min. Samples of dextran sulphate D/3, E/1, and I were each tested on blood obtained from three different rabbits.

Results obtained with three different fractions of dextran sulphate, and also the parent dextrans, are shown in Figs. 1 and 2. Dextran sulphate I (mol. wt.<10,000) did not release histamine at concentrations between 0.8 μg. and 500 μg./ml. The E/1 fraction of higher molecular weight (40,000) behaved qualitatively like the D/3 fraction (mol. wt. 440,000), but its concentration threshold for histamine release was higher, and the mean values of plasma histamine obtained at each concentration were lower, than with the latter. Results obtained with dextran showed that the molecular weight of a particular fraction was again a factor in determining its ability to initiate histamine release. The D fraction (mol. wt. 220,000) showed marked activity although its threshold concentration was higher than that of

the corresponding dextran sulphate. The E fraction (mol. wt. 22,000) showed relatively little, if any, activity, and the I fraction (mol. wt.<14,000) none at all, at concentrations between 0.8 μg. and 500 μg./ml.

When samples of blood from the same rabbit were incubated with either dextran D or dextran sulphate D/3, increasing levels of dextran D from 0.02 to 2.0 mg./ml. gave rise only to a slow increase in the level of plasma histamine, whereas dextran sulphate D/3 over the same range of concentrations caused a gradual reduction (Fig. 3).

*The Effect of Molecular Size of Dextran on Histamine Release*

Six dextran fractions were tested for their abilities to release histamine from rabbit blood cells *in vitro*. The intrinsic viscosities of the samples used ranged from 0.02 to 0.64 and represented a range of molecular weights from less than 14,000 to more than 250,000. Each fraction was incubated with 6 ml. of diluted heparinized blood from the same rabbit for 30 min. at a concentration of 100 μg./ml.

The dextran of intrinsic viscosity 0.02 did not cause a significant rise in the plasma histamine levels above those of the control samples, but increases obtained with those fractions having intrinsic viscosities ranging from 0.12 to 0.64 were significant at the P. 0.05 level (Table II).

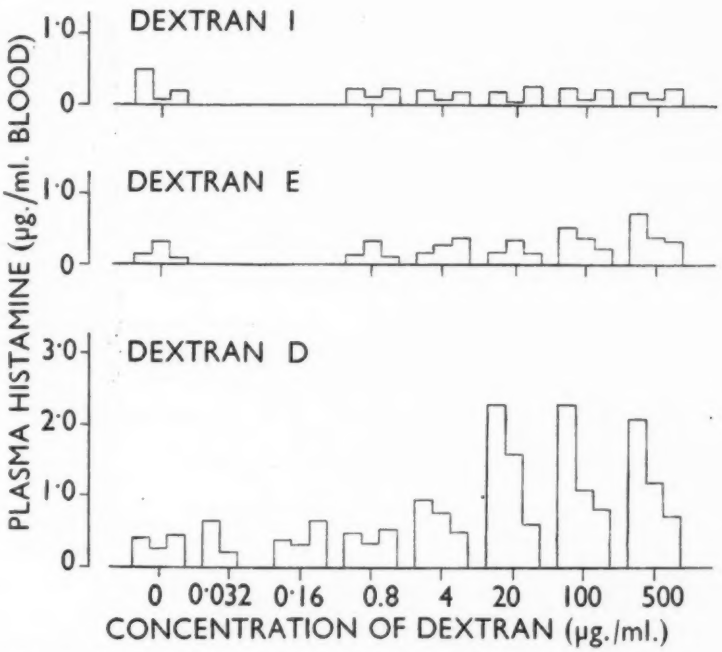


FIG. 2.—Release of histamine from rabbit blood cells by dextran. Plasma histamine levels after incubating dilute heparinized rabbit blood with varying concentrations of dextran for 30 min. Samples of dextran D, E, and I were each tested on blood obtained from three different rabbits.

TABLE II

THE *IN VITRO* RELEASE OF HISTAMINE FROM RABBIT BLOOD CELLS BY DEXTRANS OF VARYING INTRINSIC VISCOSITIES

Diluted rabbit blood was incubated with dextran 100 μg./ml. for 30 min. at 37·5° C.

| Rabbit | Plasma Histamine (μg./ml. Blood) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Intrinsic Viscosity of Dextran Fraction | | | | | |
| | | 0·02 | 0·12 | 0·25 | 0·36 | 0·44 | 0·64 |
| 38 | 0·25 | 0·32 | 0·37 | | 2·9 | | 0·81 |
| 39 | 0·58 | 0·31 | 1·4 | 3·2 | 2·7 | 3·1 | 2·2 |
| 42 | 0·69 | 0·80 | 1·4 | 2·0 | 1·8 | 2·6 | 2·1 |
| 44 | 0·25 | 0·46 | 0·67 | 2·3 | 2·5 | 1·9 | 1·2 |
| 47 | 0·17 | 0·98 | 0·64 | 0·92 | 2·0 | 1·7 | 0·64 |
| 49 | 0·43 | 0·41 | 0·76 | 1·2 | 2·7 | 3·0 | 0·62 |
| Mean values | 0·40 | 0·55 | 0·87 | 1·9 | 2·4 | 2·5 | 1·3 |

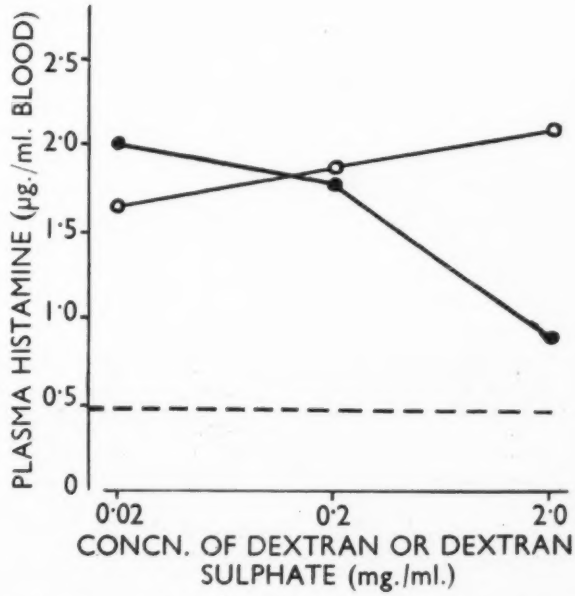


FIG. 3.—Release of histamine from rabbit blood cells. The effects of higher concentrations of dextran and dextran sulphate. Plasma histamine levels after incubating dilute heparinized rabbit blood with varying concentrations of dextran D and dextran sulphate D/3 for 30 min. Each point on the graph represents the mean of three determinations on different samples of blood. Broken line=control. Closed circles=dextran sulphate D/3. Open circles=dextran D.

Evidence that the additional histamine found in the plasma during these experiments was derived from the cellular fraction of blood, and not from the plasma or the polysaccharides themselves, was obtained by incubating dextran sulphate D/3 or dextran D with dilute rabbit plasma for 30 min. at concentrations which had previously been found effective in causing histamine release in blood. No increase in plasma histamine was obtained as a result of this procedure (Table III).

TABLE III

THE FAILURE OF DEXTRAN D OR DEXTRAN SULPHATE D/3 TO INCREASE THE LEVEL OF HISTAMINE AFTER INCUBATION WITH DILUTE RABBIT PLASMA FOR 30 MIN. AT 37·5° C. IN DUPLICATE EXPERIMENTS

| Experiment | Treatment of Dilute Rabbit Plasma During Incubation Period | Histamine Present in Dilute Rabbit Plasma μg./ml. | |
|---|---|---|---|
| 1 | Control (incubation only) | 0·15 | 0·13 |
| | Incubation with dextran sulphate D/3 0·02 mg./ml. | 0·11 | 0·16 |
| | Incubation with dextran sulphate D/3 0·5 mg./ml. | 0·12 | 0·15 |
| 2 | Control (incubation only) | 0·14 | 0·08 |
| | Incubation with dextran D 0·13 mg./ml. | 0·09 | 0·08 |
| | Incubation with dextran sulphate D/3 0·02 mg./ml. | 0·06 | 0·12 |
| | Incubation with dextran sulphate D/3 0·5 mg./ml. | 0·16 | 0·09 |

Identification of the active plasma constituent was carried out by comparing the responses of guinea-pig ileum to alternate equiactive doses of histamine and of plasma extract after the addition of mepyramine maleate (0.002 or 0.003 μg./ml.) to the bath. The same degrees of inhibition and rates of recovery were obtained with histamine and with plasma extracts from blood which had been incubated with either dextran D or dextran sulphate D/3 (Figs. 4 and 5). Since mepyramine is a highly specific inhibitor of histamine (Schild, 1947), these results make it seem likely that the active substance present in the extracts was either histamine or some closely related substance.

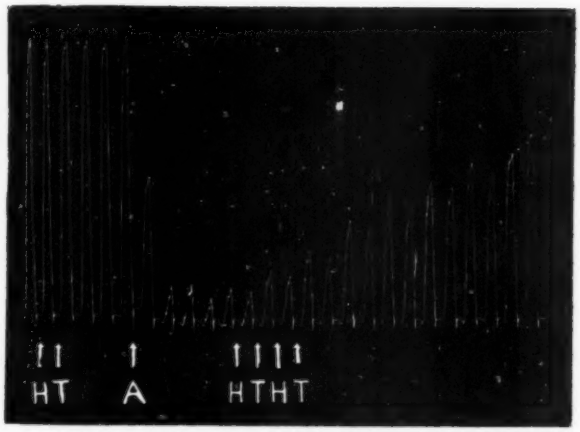


FIG. 4.—Regular contractions of isolated guinea-pig ileum suspended in Tyrode solution from alternate approximately equiactive concentrations of histamine (as acid phosphate) 0.03 μg./ml. (H), and diluted plasma extract from rabbit blood after incubation with dextran D (T). At A, mepyramine maleate 0.002 μg./ml. was allowed to remain in contact with the preparation for one minute.

*Inhibitors of Histamine Release*

When carrying out procedures involving histamine release in whole blood it was desirable to have some means of producing complete inhibition of the reaction. Sodium oxalate was known to prevent the *in vitro* release of histamine from the blood cells of sensitized rabbits by antigen (McIntire, Roth, and Richards, 1949). Its effect on the release reaction to dextran sulphate D/3 in normal rabbit blood was therefore determined. Levels of plasma histamine were compared after incubating dilute heparinized rabbit blood with dextran sulphate D/3 100 μg./ml. (*a*) alone, (*b*)

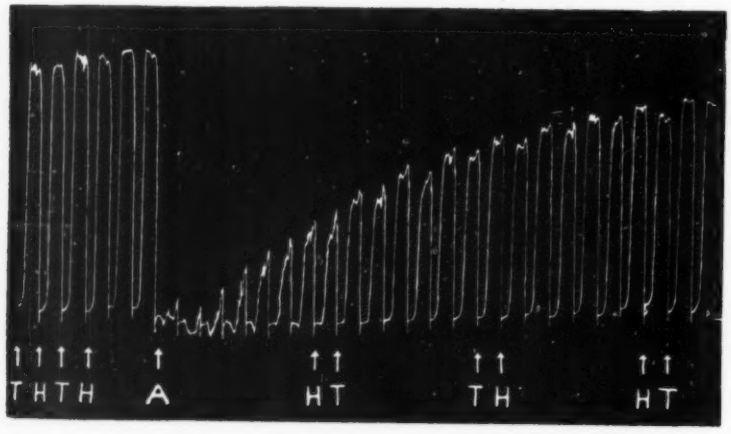


FIG. 5.—Regular contractions of isolated guinea-pig ileum suspended in Tyrode solution from alternate approximately equiactive concentrations of histamine (as acid phosphate) 0.03 μg./ml. (H), and diluted plasma extract from rabbit blood after incubation with dextran sulphate D/3 (T). At A, mepyramine maleate 0·003 μg./ml. was allowed to remain in contact with the preparation for one minute.

after the addition of sufficient sodium oxalate to give a concentration of 0.021 M during the incubation period, and (*c*) with the same quantity of sodium oxalate added at the end of the incubation period. The results are shown in Table IV. They indicate that histamine release was prevented completely by the sodium oxalate when added before the dextran sulphate D/3, but that the same quantity added after histamine release had taken place

TABLE IV

INHIBITORY EFFECT OF SODIUM OXALATE 0·021M ON THE RELEASE OF HISTAMINE FROM RABBIT BLOOD CELLS INCUBATED WITH DEXTRAN SULPHATE D/3 100 μG./ML.

| Rabbit | Plasma Histamine (μg./ml. Blood) | | | |
|---|---|---|---|---|
| | Control Incubation with Normal Saline | Incubation with Dextran Sulphate D/3 100 μg./ml. | Sodium Oxalate 0·021M Added Before Incubation with Dextran Sulphate D/3 100 μg./ml. | Sodium Oxalate 0·021M Added After Incubation with Dextran Sulphate D/3 100 μg./ml. |
| 35 | 0·29 | 2·4 | 0·38 | 2·3 |
| 39 | 0·64 | 3·0 | 0·13 | 3·1 |
| 33 | 0·55 | 1·5 | <0·06 | 1·2 |
| 40 | 0·57 | 1·6 | 0·22 | 1·6 |
| 32 | 0·44 | 3·7 | 0·14 | 3·7 |
| Mean values | 0·50 | 2·44 | <0·19 | 2·38 |

did not interfere with the amount of histamine found after extraction and assay. Since the values for plasma histamine obtained when sodium oxalate was added to blood before the dextran sulphate D/3 were lower than those obtained with untreated blood, it seems likely that the sodium oxalate in addition to inhibiting the histamine release due to dextran sulphate D/3 also prevented a non-specific release of histamine from the cells during the incubation period. On the basis of these results, sodium oxalate was used in further experiments to stop the release of histamine as required.

Dextran sulphate of high molecular weight was found to possess a dual action, behaving as an activator of the histamine-release mechanism in low concentrations and as an inhibitor at higher concentrations. This fact indicated that fractions of low molecular weight that were incapable of releasing histamine might behave as inhibitors. This hypothesis was tested by incubating samples of dilute heparinized blood containing either dextran I or dextran sulphate I with dextran sulphate D/3 100 μg./ml. Dextran I at concentrations of 100 μg./ml. and 500 μg./ml. failed to cause any reduction in the amount of histamine released, but dextran sulphate I gave mean reductions of 21% and 60% of plasma histamine respectively when the same concentrations were used (Table V).

TABLE V

EFFECTS OF DEXTRAN SULPHATE I AND DEXTRAN I ON THE RELEASE OF HISTAMINE FROM RABBIT BLOOD CELLS INCUBATED WITH DEXTRAN SULPHATE D/3 100 μG./ML.

| Rabbit | Plasma Histamine (μg./ml. Blood) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Dextran Sulphate D/3 100 μg./ml. | Dextran Sulphate D/3 100 μg./ml. Plus Dextran I | | Dextran Sulphate D/3 100 μg./ml. Plus Dextran Sulphate I | |
| | | | 100 μg./ml. | 500 μg./ml. | 100 μg./ml. | 500 μg./ml. |
| 31 | 0·63 | 1·4 | | | 0·69 | 0·30 |
| 31 | 0·27 | 1·2 | 1·3 | 1·2 | 0·62 | 0·22 |
| 26 | 0·57 | 3·7 | 3·2 | 3·4 | 2·8 | 1·2 |
| 38 | 1·25 | 3·0 | 3·4 | 3·5 | 3·5 | 2·1 |
| 39 | 0·56 | 2·9 | 3·8 | 3·8 | 2·9 | 1·7 |
| Mean percentage reduction of plasma histamine .. | | | −10 | −10 | 21 | 60 |

These results suggested that sulphation of the dextran was necessary in order to obtain compounds capable of inhibiting the histamine release reaction, and it seemed possible that sulphation of other polysaccharides would give rise to the same properties. Recent work (Ricketts, 1952a) showed that sulphuric esters of polysaccharides of low molecular weight possessed anticoagulant activity *in vitro*. The effect of one of these, maltotriose sulphate, was compared with that of heparin in its ability to inhibit the histamine-releasing action of dextran sulphate D/3 in rabbit blood. The results are shown in Table VI. On a weight for weight basis, maltotriose sulphate was more effective as an inhibitor of histamine release than heparin; the mean reduction in plasma histamine after heparin 500 μg./ml. was 34%, and that after maltotriose sulphate 500 μg./ml. was 77%.

TABLE VI

INHIBITORY EFFECTS OF HEPARIN 500 μG./ML. AND MALTOTRIOSE SULPHATE 500 μG./ML. ON THE RELEASE OF HISTAMINE FROM RABBIT BLOOD CELLS INCUBATED WITH DEXTRAN SULPHATE D/3 100 μG./ML.

| Rabbit | Plasma Histamine, μg./ml. of Blood | | | |
|---|---|---|---|---|
| | Control | Dextran Sulphate D/3 100 μg./ml. | Dextran Sulphate D/3 100 μg./ml. Preceded by Heparin 500 μg./ml. | Dextran Sulphate D/3 100 μg./ml. Preceded by Maltotriose Sulphate 500 μg./ml. |
| 14 | 0·12 | 1·9 | 1·4 | 0·28 |
| 43 | <0·14 | 0·78 | 0·17 | 0·12 |
| 2a | <0·09 | 1·6 | 1·2 | 0·37 |
| 4a | <0·09 | 1·0 | 1·0 | 0·40 |
| 30 | 0·16 | 1·8 | 1·1 | |
| 31 | 0·18 | 0·97 | 0·67 | |
| Mean percentage reduction of plasma histamine .. | | | 34 | 77 |

In order to confirm that dextran sulphate I and heparin were not interfering with the extraction or assay of histamine, four samples of dilute heparinized blood from the same rabbit were incubated with: (*a*), sodium oxalate to prevent histamine release, followed by dextran sulphate D/3; (*b*), dextran sulphate D/3 followed after incubation by sodium oxalate; (*c*), dextran sulphate D/3 followed after incubation by sodium oxalate, and then by dextran sulphate I; and (*d*), dextran sulphate D/3 followed after incubation by sodium oxalate and then by heparin. In each sample the final molarity of sodium oxalate was 0.019, and dextran sulphate D/3 100 μg./ml. was present during the incubation period. The concentrations of dextran sulphate I and heparin were similar to those used in previous experiments summarized in Tables V and VI. The results are presented in Table VII. Comparison of the plasma histamine values given in columns B, C, and D makes it clear that neither dextran sulphate I nor heparin caused any reduction in the level of plasma histamine, so that the inhibitory effects on histamine release previously obtained could not be attributed to interference with the extraction or assay of plasma histamine.

TABLE VII

FAILURE OF HEPARIN AND DEXTRAN SULPHATE I TO INTERFERE WITH THE EXTRACTION AND ASSAY OF HISTAMINE RELEASED FROM RABBIT BLOOD BY INCUBATION WITH DEXTRAN SULPHATE D/3

| Rabbit | Plasma Histamine (μg./ml. Blood) | | | |
|---|---|---|---|---|
| | Blood Plus Sodium Oxalate, then Dextran Sulphate D/3 (100 μg./ml.) Before Incubation<br>A | Blood Plus Dextran Sulphate D/3 (100 μg./ml.) Before Incubation, then Sodium Oxalate After Incubation<br>B | Blood Plus Dextran Sulphate D/3 (100 μg./ml.) Before Incubation, then Sodium Oxalate and Dextran Sulphate I After Incubation<br>C | Blood Plus Dextran Sulphate D/3 (100 μg./ml.) Before Incubation, then Sodium Oxalate and Heparin After Incubation<br>D |
| 35 | 0·38 | 2·3 | 1·9 | 2·5 |
| 39 | 0·13 | 3·1 | 3·0 | 2·9 |
| 33 | 0·06 | 1·2 | 1·2 | 1·2 |
| 40 | 0·22 | 1·6 | 1·5 | 1·5 |
| 32 | 0·14 | 3·7 | 3·7 | 3·5 |
| Mean values | 0·19 | 2·38 | 2·26 | 2·32 |

## DISCUSSION

These results show that certain samples of both dextran and dextran sulphate will release histamine, or some closely related substance, from the cells of rabbit blood *in vitro*. Of interest is the observation that dextran and dextran sulphate show differences in their behaviour as releasing agents at high concentrations. The quantity of histamine released by a particular fraction of either dextran or dextran sulphate depends upon its concentration. With dextran, the molecular weight of the fraction used is important in determining its activity.

The influence of concentration and molecular weight of dextran sulphate on its behaviour in biological reactions has been previously described by Walton (1952), who stressed the points of similarity between non-specific precipitation of fibrinogen by dextran sulphate at physiological *p*H and the precipitin reaction between antigen and antibody. It is interesting to note that the behaviour of dextran sulphate as a histamine-releasing agent and as a precipitant of fibrinogen has the following features in common. (1) Release of histamine and precipitation of fibrinogen occur only with samples of dextran sulphate above a certain molecular weight. (2) Optimum concentration of dextran sulphate is necessary for maximum histamine release or fibrinogen precipitation, since an excess inhibits either reaction. (3) Precipitation of fibrinogen and release of histamine are inhibited by heparin or by dextran sulphate of low molecular weight. Whilst these similarities may be incidental they would not be entirely unexpected if interaction between dextran sulphate and a protein constituent of the plasma was a prerequisite for the initiation of histamine release.

Since dextrans are polymers consisting of glucose molecules joined through 1:6 α glucoside linkages as branched chains (Ricketts and Walton, 1952), the intrinsic viscosity of any particular sample gives an indication of its molecular weight and the size of its constituent molecules. The dextrans used in these experiments were polydisperse, and therefore only the average molecular weights of the different fractions were known. Equal concentrations of dextrans having intrinsic viscosities from 0.12 to 0.44 gave rise to graded increases in the quantities of histamine released under the same conditions, in spite of the fact that increasing values of intrinsic viscosity were accompanied by parallel reductions of molarity.

In what way molecular size influences the histamine-release mechanism is not clear. The actual shape of the molecule may be of importance, and activation of the release mechanism may be directly related to molecular size, or may depend simply upon the presence of molecules above a certain critical size. In this respect, the smaller release of histamine obtained when using

dextran of intrinsic viscosity 0.64, as compared with those with values of 0.36 or 0.44, may have been owing to the smaller number of molecules present.

It seems probable that molecular weight is also a factor in determining whether a fraction of dextran sulphate will cause histamine release. No attempts were made to confirm this, since by analogy with the anticoagulant action of dextran sulphate (Ricketts, 1952b) it was thought that the degree to which a particular fraction had been sulphated might also prove to be an additional factor in modifying activity. Those samples which were available at the time had not been sulphated to the same extent.

Apart from confirming that the histamine released into rabbit plasma was derived from the cells, and not from the releasing agents themselves, or as a result of some action on the plasma constituents, no attempts were made to determine its origin. It seems likely, however, that it was derived from the platelets, which are considered to be the main carriers of histamine in rabbit blood (Code, 1952).

Those dextran sulphates effective in causing histamine release have also been reported to cause clumping of platelets *in vitro* and *in vivo* (Walton, 1954). The possibility exists, therefore, that consequent damage to platelets could be sufficient to cause liberation of their histamine. According to Rocha e Silva (1950), platelets form aggregates, and later disintegrate, during anaphylactic shock in the rabbit; this breakdown of platelets can be correlated with release of histamine. This is in contrast to the stable aggregations of platelets which form after the intravenous injections of various polysaccharides.

In the present experiments the fact that, above a certain level, increasing concentrations of dextran sulphate D/3 gave rise to decreasing levels of released histamine points to the presence in plasma or cells of some mechanism leading to histamine release which can be either reversed or blocked by the dextran sulphate. Such mechanism could be entirely independent of any direct damaging effect which a dextran or dextran sulphate may have on platelets.

It is rather striking that substances which inhibit the histamine release in rabbit blood by one particular agent are frequently effective against a variety of others. Sodium oxalate, in addition to preventing histamine release by dextran sulphate, has also been used to inhibit that due to a variety of primary amines, quaternary pyridinium esters, morphine (McIntire, Roth, and Sproull, 1951), to antigen-antibody reactions in normal rabbit blood (McIntire, Roth, and Sproull, 1950), and to addition of the specific antigen to blood from a sensitized rabbit—a property shared by heparin (Dragstedt, Wells, and Rocha e Silva, 1942; McIntire, Roth, and Richards, 1949). Heparin has also been shown to inhibit histamine release in normal rabbit blood by proteose and trypsin (Dragstedt, Wells, and Rocha e Silva, 1942). There were also indications that it would reduce the quantity of histamine released from the cells of normal rabbit blood incubated with washed antigen-antibody complex—a property shared by dextran sulphate I (Haining, 1953).

That the same inhibitors are effective in influencing the release of histamine due to such a variety of apparently dissimilar agents suggests that the number of pathways leading to histamine release in rabbit blood may be very restricted.

## Summary

1. Certain fractions of dextran and of dextran sulphate were found to cause the release of histamine when incubated with rabbit blood.

2. With dextran, histamine release was dependent upon concentration and molecular weight. Samples having molecular weights between 22,000 and 1,000,000 were effective, but not those below 14,000.

3. Samples of dextran sulphate with molecular weights below 10,000 did not release histamine, but those with values from 40,000 to 440,000 showed a graded ability to do so. Dextran sulphate differed from dextran in that an optimum concentration was necessary in order to obtain maximum histamine release.

4. The release of histamine in rabbit blood by dextran sulphate D/3 was inhibited by sodium oxalate, dextran sulphate, heparin, and maltotriose sulphate. The importance of the ester sulphate groupings in this respect was shown by the fact that dextran (m.w.$<$14,000) was ineffective.

I wish to express my thanks to Professor A. C. Frazer for his continued interest in this work and for provision of the necessary facilities, and to Dr. P. B. Marshall for his sympathetic direction and advice.

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# THE SPASMOLYTIC PROPERTIES OF THIOBENZILIC ACID ESTERS AND RELATED COMPOUNDS

BY

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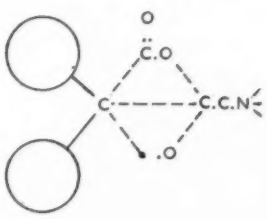
In the absence of any clear understanding of the causes underlying the clinical occurrence of spasm in smooth muscle, the search for agents by which it might be relieved has always been pursued empirically. There are conditions, such as asthma, in which drugs reducing spasm by parasympatholytic action may be undesirable, on account of side effects, and for which specific antihistamines are not satisfactorily effective. For such conditions a non-specific inhibitor of smooth muscle tonus and action might be the type of drug required. Papaverine exerts this kind of spasmolytic effect, but has certain disadvantages. To investigate this problem a series of compounds was screened for papaverine-like activity, using the commonly adopted criterion of reduction of barium-induced spasm in isolated intestine.

Spasmolytic activity appears to be particularly associated with certain molecular structures (Blicke, 1944; Bovet and Bovet-Nitti, 1948; Burtner, 1951; Bergel and Parkes, 1952). Atropine-like antagonism of acetylcholine on isolated strips of intestine is, in general, associated with a structure consisting of a short chain of atoms linking a tertiary or quaternary nitrogen atom to a carbon atom bearing aromatic or other ring systems, as may be schematically represented thus:




Compounds of this type may show, in addition, activity in reducing spasm produced by other agents such as histamine or barium chloride.

The substances tested were synthesized in the chemical laboratories of the Research Department, Roche Products Ltd., and consisted of a series of basic esters of thiobenzilic acid,

$$Ph_2C(OH)CO.S.(CH_2)_nNRR',$$

and derivatives, described by Morrison and Konigstein (1949), and a few derivatives of benzilic acid esters (Morrison, Konigstein, and Cohen, 1950), together with ethers and thioethers of the type

$$Ph_2C(OH)CH_2O(\text{or S})\,(CH_2)_nNRR'.$$

## METHODS

*Isolated Guinea-pig Ileum.*—Lengths of guinea-pig ileum were suspended in a 30 ml. bath containing oxygenated Tyrode solution at 37° C. (composition: NaCl 0.8%, KCl 0.02%, $CaCl_2$ 0.01%, $MgCl_2$ 0.01%, $NaH_2PO_4$ 0.005%, $NaHCO_3$ 0.1%, glucose 0.1%). Addition of test drugs was regulated automatically by a device similar to that recommended by Schild (1947); two baths were usually employed simultaneously.

Solutions of drugs were diluted with distilled water and added to the bath in volumes of not more than 1 ml. Test substances were allowed 2 min. contact with the gut before the addition of the spasmogenic drug, which remained in the bath for a further 15 sec., except for barium chloride and nicotine, which usually required 30 sec. for full effect. After each spasmogenic drug the bath was drained and refilled twice at an interval of 1 min., the cycle being completed by a resting period usually of 4 min., but up to 15 min. where necessary, as, for example, when using nicotine. Responses of the gut were recorded by a lever with a frontal writing point.

Each substance tested as an antagonist was compared directly with a standard drug for action against a repeated dose of spasmogenic agent chosen to give a suitable submaximal response. Usually a four-point assay routine of the type described by Schild (1947) was used. The number of assays varied with the apparent activity of the compound, and an activity of interesting magnitude would be accorded two or three assays on each of several lengths of gut, preferably from more than one animal.

Drugs used to stimulate the gut were acetylcholine chloride, histamine acid phosphate, barium and potassium chlorides, and nicotine hydrochloride. The

drugs chosen as standard antagonists were atropine sulphate, mepyramine hydrochloride, and, against both metallic salts and nicotine, papaverine hydrochloride.

Rabbit and rat ileum were also used to assay anti-Ba activity in the same way as described for guinea-pig ileum; rabbit ileum was further used to assay activity in reducing spontaneous motility in comparison with papaverine.

*Isolated Rat Uterus.*—This organ was suspended in oxygenated Locke's solution at 36° C. and stimulated by barium chloride. Substances were assayed for antagonistic activity in comparison with papaverine.

*Isolated Air-perfused Lung.*—The preparation used was similar to that described by Arunlakshana and Schild (1950). Guinea-pig lungs, removed from animals stunned and bled, were perfused via the pulmonary artery with the fluid described by McDowall and Thornton (1930) at 36° C. under a constant pressure of 15 cm. Air was led into the trachea at a constant rate and escaped through scarifications in the lung surface. The pressure set up in maintaining the rate of air flow was recorded. The bronchial vessels were constricted by perfusing with fluid containing barium chloride. Drugs were tested for antagonism either by adding them to the fluid to be perfused, in the required dilution, or by injecting doses into the stream immediately above the cannula.

*Isolated Tracheal Muscle.*—The preparation used was the tracheal chain, first described by Castillo and de Beer (1947). This was suspended in Thornton's fluid or in Krebs-Henseleit fluid at 37° C. Dr. D. F. Hawkins has found that if the bath is aerated with oxygen the *p*H rises to 8.4 and the guinea-pig tracheal chain takes on a high resting tonus; this has been used in the detection of relaxant activity. When the gas bubbling through the fluid contains 5% $CO_2$ the *p*H falls to 7.4 and the guinea-pig preparation is fully relaxed, as is that of the rabbit under either condition. These chains can be stimulated by spasmogenic agents and have been used to determine spasmolytic activity.

*Coronary-perfused Heart.*—The Langendorff preparation of the rabbit heart was perfused with Locke solution at 37° C., under a constant pressure of 120 cm. fluid. Beat and coronary flow were recorded. Solutions of test substances were diluted in Locke's solution and injected slowly in small volumes above the aortic cannula. Coronary dilator activity was assayed in comparison with papaverine.

Substances were supplied either as hydrochlorides and dissolved in water for testing, or as base and dissolved with the aid of equivalent hydrochloric acid.

## Results

*Isolated Guinea-pig Ileum.*—The results of assays upon this organ are given in Table I, where activities are expressed relative to that of the standard antagonist used for comparison. Among the series of thioesters studied was the thio-analogue of "Trasentin" (β-diethylaminoethyl diphenylthioacetate, Ro 3-0235), the spasmolytic activity of which was reported by Dupré, Lévy and Tchoubar (1946) and also by Ramsay and Richardson (1947).

In this series considerable activities in antagonizing ACh, Ba and K were found among the thioesters of benzilic acid. Comparison of the diethylaminoethyl thioesters Ro 3-0235, Ro 3-0226 and Ro 3-0297 shows that substitution of the hydroxyl group by hydrogen reduced atropine-like activity, whereas introduction of a methyl group considerably reduced ability to anatagonize both ACh and Ba. Comparison of the pairs of compounds Ro 3-0290 and Ro 3-0298, Ro 3-0299 and Ro 3-0303, Ro 3-0305 and Ro 3-0306 further demonstrates the dependence of activity upon the hydroxyl group. Etherification of this group also reduced activity—compare, e.g., Ro 3-0292 with Ro 3-0290.

Activities among the aminoalkanethiol esters of benzilic acid appeared to be influenced both by the substituents on the nitrogen atom and by the length and character of the alkylene chain. The relationships may be seen from Table II. In the aminoethylthiol esters studied, anti-ACh activity was high with the diethylamino- member and even higher with the dimethylamino- (Ro 3-0378, Table I), but was progressively reduced in the piperidino- and morpholino- compounds. A similar tendency appeared to exist in the series derived from aminopropylthiol. The effect of chain-length upon anti-ACh activity showed differences between the diethylamino- and the piperidino- series; activity in the former decreased progressively with increase in chain-length, whereas in the latter it was low, rising suddenly to twice that of atropine with the piperidinohexyl member.

The more active compounds are very potent Ba antagonists, some being effective at dilutions of 1 in 50 million. Many of the mean activities, however, are associated with considerable variance; a number of the compounds—particularly the most active—gave widely different activities at different periods. For these and other reasons to be discussed later it is not possible to ascribe significance to any differences or trends which may appear to follow changes in structure, as is possible for anti-ACh activity.

Activities in antagonizing the effects of K ions also did not fall into a clearly defined pattern in this series. None of the compounds showed any appreciable antihistamine activity.

## TABLE I

SPASMOLYTIC ACTIVITIES OF VARIOUS BENZILIC ACID DERIVATIVES ON ISOLATED GUINEA-PIG ILEUM

Anti-acetylcholine activities assayed against atropine, anti-barium and anti-potassium activities against papaverine, and anti-histamine activities against mepyramine

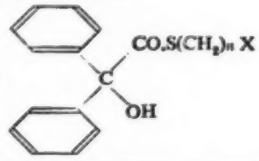
| Ro Series No. | Formula | | | Activity against Spasm due to: ACh (Atropine = 100) | $BaCl_2$ (Papaverine = 1) | KCl | Histamine (Mepyramine = 100) |
|---|---|---|---|---|---|---|---|
| | | | | $Ph_2:C.CO.S.CH.CH_2.R^3$ | | | |
| | | | | $R^1$ $R^2$ | | | |
| *Thioesters* | $R^1$ | $R^2$ | $R^3$ | | | | |
| 3–0235 | H | H | $NEt_2$ | 20±10 (5)† | 150±87 (5)*; 27±9 (5) | 6·2±1·3 (5) | 0·5 (3) |
| 3–0226 | OH | H | $NEt_2$ | 112 (2) | (5150±1150 (5)*; 73±30 (3) | 17·5±12 (3) | 0·15 |
| 3–0297 | Me | H | $NEt_2$ | 2·65 (3) | 13·3±1·3 (3) | 3·8 (3) | 0·45 (3) |
| 3–0378 | OH | H | $NMe_2$ | 163±9 (5) | 2·32±0·6 (3) | 1·96 (2) | — |
| 3–0348 | OH | H | | 2·7±2·4 (5) | 90±20 (4) | 10–200 (7) | <0·01 |
| 3–0368 | OH | H | | 0·025 (3) | 0·5 (2) | 0·36 (3) | 0·005 (3) |
| 3–0296 | OH | H | $CH_2NEt_2$ | 65±4·2 (3) | 875±410 (5)*; 23·5±7 (3) | 310 (2) | 0·03 |
| 3–0290 | OH | H | | 6·2±1·6 (3) | 91±27 (8)*; 10·3±3·4 (5) | 70±21 (5) | 7·5 |
| 3–0292 | OEt | H | | 1 | 3·2±1·2 (5) | 5 | 0·15 |
| 3–0298 | Me | H | | 0·2 | 3·3±0·8 (5) | 5·4±0·6 (5) | 0·1 |
| 3–0299 | OH | H | | 1·12 (2) | 28±3·4 (4) | 5·8±0·64 (3) | <0·01 |
| 3–0303 | Me | H | | 0·12 (2) | 1·35 (2) | 1·5 | 0·02 |
| 3–0305 | OH | Me | | 2·7 (4) | 10±4·7 (5) | 12·5 | 0·01 |
| 3–0306 | Me | Me | | 0·2 | <1 | 1 | <0·1 |
| 3–0341 | OH | H | $(CH_2)_2NEt_2$ | 1·1 (8) | 7·75±6 (6) | 5 (3) | 0·005 (5) |
| 3–0342 | OH | H | $(CH_2)_3NEt_2$ | 0·22 (5) | 4±0·6 (4) | 10·8±5·65 (8) | 0·03 (4) |
| 3–0320 | OH | H | | 6±1·5 (3) | 50·5±25 (4)*; 17·2±8 (4) | 41±10 (4) | 0·1 (2) |
| 3–0367 | OH | H | | 200±95 (12) | 2·2 (2) | 1·5 (2) | 0·025 (2) |
| | | | | $Ph_2:C.CO.O.CH_2.CH_2.NEt_2$ | | | |
| *Benzilic Acid Derivatives* | $R^1$ | | | $R^1$ | | | |
| 3–0256 | OH | | | 50 (2) | 20 | 50 | — |
| 3–0131 | $OCH_2CH_2NMe_2$ | | | 0·5 (4) | 7·5 (2) | 12 (2) | 0·2 |
| | | | | $Ph_2:C.CH_2.A.CH_2.CH_2.R^2$ | | | |
| *Ethers* | $R^1$ | A | $R^2$ | $R^1$ | | | |
| 3–0327 | OH | O | $NMe_2$ | 0·5 (5) | 0·5 (4) | 4–10 (2) | 0·02 |
| 3–0328 | OH | O | $NEt_2$ | 0·5 (3) | 3·3±0·72 (4) | 9·15±1·9 (5) | 0·01 |
| 3–0354 | $OCH_2CH_2NMe_2$ | O | $NEt_2$ | 0·18 (2) | 1·8 (2) | 4·2 (3) | 0·15 (2) |
| 3–0380 | OH | S | $NMe_2$ | 1·2±0·17 (3) | 9·3±7·8 (3) | 0·75 (2) | 0·05 (2) |
| 3–0326 | OH | S | $NEt_2$ | 1·85 (2) | 79·5±12·5 (11) | 19·5±7·5 (5) | 0·1 (2) |
| 3–0373 | OH | S | $CH_2NEt_2$ | 4·3±2·2 (3) | 0·46±0·02 (4) | 14·2±8·3 (3) | 0·025 |
| 3–0353 | OH | $SO_2$ | $NEt_2$ | 0·4 (7) | 1·5±0·5 (6) | 1·15 (2) | 0·001 (3) |

*Values obtained at different periods (see text). † Mean values ± S.D The figures in parentheses give the number of assays from which the mean is derived.

TABLE II
ACTIVITIES OF THIOESTERS ON ISOLATED GUINEA-PIG ILEUM

Values as in Table I

General formula




| | X = —NEt$_2$ | | | | X = —N⬡ | | | | X = —N⬡O | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| n | Ro Series No. | Anti-ACh | Anti-Ba | Anti-K | Ro Series No. | Anti-ACh | Anti-Ba | Anti-K | Ro Series No. | Anti-ACh | Anti-Ba | Anti-K |
| 2 | 3-0226 | 112 | 5150±1150*<br>73±30 | 17·5±12 | 3-0348 | 2·7±2·4 | 90±20 | 10–200 | 3-0368 | 0·025 | 0·5 | 0·36 |
| 3 | 3-0296 | 65±4·3 | 875±410*<br>23·5±7 | 310 | 3-0290 | 6·2±1·6 | 91±27*<br>10·3±3·4 | 70±21 | 3-0299 | 1·12 | 28±3·4 | 5·8±0·64 |
| 4 | 3-0341 | 1·1 | 7·75±6 | 5 | | | | | | | | |
| 5 | 3-0342 | 0·22 | 4±0·6 | 10·8±5·65 | 3-0320 | 6±1·5 | 50·5±25*<br>17·2±8 | 41±10 | | | | |
| 6 | | | | | 3-0367 | 200±95 | 2·2 | 1·5 | | | | |

*Values obtained at different periods.

Ro 3-0256 (β-diethylaminoethyl benzilate) was first tested by Halpern (1938) and later by Lehmann and Knoefel (1942), by Ing, Dawes, and Wajda (1945), by Buchel, Lévy, and Pernot (1948) and by Lands (1951). The value reported here for anti-ACh activity is similar to those found in tests on rabbit intestine by Halpern, by Lehmann and Knoefel, and by Lands, though the values reported by previous workers for anti-Ba activity are far less than those reported here. Ro 3-0256 was included in this series for comparison with the corresponding thioester Ro 3-0226; Table I shows that the thio-linkage may be associated with somewhat higher activity against ACh than the normal ester linkage.

The compound Ro 3-0131 (β-diethylaminoethyl diphenyl (β-dimethylaminoethoxy)-acetate) was reported upon by Forbes and Marshall (1951). The anti-Ba activity of Ro 3-0131 reported here, relative to that of papaverine, was of the same order as that calculated from the $pA_2$ values given by these authors. Table I shows that etherification of the benzilic hydroxyl group reduced all types of spasmolytic activity.

The substitution of the ester linkage by an ether linkage reduced all activities (Table I : compare Ro 3-0256 with Ro 3-0328, and Ro 3-0131 with Ro 3-0354). The thioether Ro 3-0326 and the corresponding thioester Ro 3-0226, however, appeared to possess similar activity against both Ba and K although the ether lacked anti-ACh activity. Similar reduction in atropine-like activity distinguished the thioether Ro 3-0380 from the thioester Ro 3-0378. Oxidation of the sulphur linkage in Ro 3-0326 to form the compound Ro 3-0353 decreased all activities markedly.

*Isolated Ileum of Rabbit and Rat.*—A number of substances shown in Table I were also tested on isolated rabbit ileum, principally for activity in reducing spontaneous motility and antagonizing spasm due to $BaCl_2$. A few were also tested against Ba spasm on isolated rat ileum. The results are given in Table III. No direct correlation could be detected between the activities of most of the compounds and those obtained with guinea-pig ileum. However, some of the figures relating to anti-Ba effects for the rabbit correspond with those for the rat.

TABLE III
ACTIVITIES ON ISOLATED ILEUM OF RABBIT AND RAT
(Papaverine = 1)

| Ro Series No. | Rabbit Ileum | | Rat Ileum |
|---|---|---|---|
| | Against Spontaneous Motility | Against Ba Spasm | Against Ba Spasm |
| 3-0226 | 74·0±41 (5)* | 3·0± 1·0 (3) | 7·5± 4 (6) |
| 3-0235 | 9·3± 7·8 (7) | 2·2± 0·8 (5) | 21·0± 7 (5) |
| 3-0290 | 6·0± 3·0 (6) | 38·5±12·0 (4) | 38·0±11 (5) |
| 3-0296 | 6·0 (2) | 3·5 | — |
| 3-0299 | 6·5 | 0·75 (2) | — |
| 3-0305 | 0·7 (2) | 2·5± 1·3 (3) | — |
| 3-0320 | 17·0± 6·5 (4) | 16·0± 0·5 (4) | 20·0± 6 (7) |
| 3-0326 | 2·5± 1·07 (7) | 5·0± 2·2 (6) | — |
| 3-0373 | 1·6 (2) | — | — |

* Mean values ± S.D. The figures in parentheses give the number of assays from which the mean is derived.

*Other Isolated Organ Preparations.*—A number of compounds were tested on other preparations: for relaxant activity on the tracheal chain of the guinea-pig; for anti-Ba activity on the rat uterus, the tracheal chain of the rabbit and the isolated air-perfused lung of the guinea-pig; and for coronary dilator activity on the Langendorff preparation of rabbit and guinea-pig heart. The results are collected in Table IV.

TABLE IV
ACTIVITIES ON VARIOUS ISOLATED ORGAN PREPARATIONS
(Papaverine = 1)

| | |
|---|---|
| *Tracheal chain, guinea-pig, pH 8·4, relaxant activity* | |
| Ro3–0226 0·01 | Ro3–0326 0·015 |
| Ro3–0299 0·02 | Ro3–0348 0·01 |
| *Tracheal chain, rabbit, pH 7·4, reduction of Ba spasm* | |
| Ro3–0299 0·5 | Ro3–0348 1·0 |
| Ro3–0320 0·1 | Ro3–0368 0·5 |
| Ro3–0326 0·12 | |
| *Air-perfused guinea-pig lung, reduction of effects of Ba perfusion* | |
| Ro3–0290 1·0 | Ro3–0326 1·0 |
| Ro3–0320 ca. 1 | |
| *Langendorff preparation, coronary dilator activity* | |
| Ro3–0226 0·15 (guinea-pig) | Ro3–0296 1·0(guinea-pig) |
| Ro3–0235 1·0 ,, 1·0 (rabbit) | Ro3–0299 0·25 ,, |
| Ro3–0290 1·0 ,, 0·7 ,, | Ro3–0320 1·5 ,, |
| Ro3–0292 0·4 (rabbit) | Ro3–0326 0·25 ,, |
| *Rat uterus, reduction of Ba spasm* | |
| Ro3–0226 1·0 (3 assays) | Ro3–0326 0·2 |
| Ro3–0320 1·0 (2 ,, ) | |

The activities of the compounds in these tests were but a fraction of those on intestine, except that, in reducing ACh spasm in the guinea-pig tracheal chain, Ro 3-0226 was as active as atropine, a result similar to that with guinea-pig ileum.

*Further Tests on Guinea-pig Ileum.*—Because the action of barium upon intestine is at least partly mediated by stimulation of ganglion cells (Ambache, 1949; Feldberg, 1951), it seemed possible that the inhibition of barium spasm in guinea-pig ileum might be exerted upon ganglia. Some of the compounds were therefore compared with papaverine against the stimulant action of nicotine hydrochloride on isolated guinea-pig ileum. Re-determinations of anti-Ba activity were also made for two of the compounds, Ro 3-0235 and Ro 3-0226—which, as Table I shows, gave different results at different periods—and the values obtained were only a fraction of any found earlier. Inquiry revealed that the animals used for these tests, and those with which the earlier tests were performed, were from different sources. Anti-barium and anti-nicotine activities of five compounds, four of which were highly active in earlier tests and one of which appeared to be weak, were therefore re-determined on lengths of ileum from guinea-pigs of two different strains. In addition, a few animals, obtained from the same source as those in the earlier tests, were used to determine the anti-Ba activity of Ro 3-0226; similar determinations were also made for this compound on gut from animals of yet a fourth strain. The results are given in Table V. The activities, relative to papaverine, were much lower against both barium and nicotine with animals of strain II than were those originally obtained with strain I. The values with strain III, however, were of the order of the original results. Ro 3-0226 was a potent Ba antagonist with animals of strain IV and of the strain corresponding to strain I, secured later than the original group. Similar differences were found for activities against nicotine in strains II and III, in which some of the compounds were more potent against nicotine than against Ba. Indeed, the most active of the compounds, Ro 3-0348, was effective in reducing responses to nicotine in dilutions of the order of 1 in $10^9$. It should be emphasized that the differences in relative activity were due entirely to differences in activity of the test compounds, papaverine being consistently effective in dilutions of

TABLE V
STRAIN DIFFERENCES IN ACTIVITIES AGAINST BARIUM AND NICOTINE ON ISOLATED GUINEA-PIG ILEUM
(Papaverine = 1)

| Ro Series No. | Against Barium | | | | | Against Nicotine | |
|---|---|---|---|---|---|---|---|
| | Strain I* (Original) | Strain II | Strain III | Strain IV | Strain I (Later) | Strain II | Strain III |
| 3–0226 | 5150±1150 (5)<br>73±30 (3) | 11·8±4·2 (15) | 88·5±52 (9) | 55±32 (4) | 161±75·5 (12) | 218±67 (8) | 850±650 (12) |
| 3–0235 | 150±87 (5)<br>27±9 (5) | ca. 5 | 86±39 (10) | | | ca. 5 | 57±37 (13) |
| 3–0326 | 79·5±12·5 (11) | ca. 5 | 118±64 (11) | | | 16±5 (10) | 85±60 (6) |
| 3–0348 | 90±20 (4) | ca. 1 | 148±96·5 (10) | | | 870±166 (9) | 2750±1300 (10) |
| 3–0367 | 2·2 (2) | ca. 2 | 31±8·8 (7) | | | ca. 20 | 70±25 (6) |

* Values from Table I.

the order of 1 in a million. Gut from all strains contracted in response to the same order of concentration of stimulant agents.

Feldberg (1951) showed that hexamethonium, in a dilution of 1 in 150,000, antagonized the stimulant actions of Ba and nicotine on the isolated guinea-pig ileum. However, with gut from guinea-pigs of Strain II, hexamethonium was ineffective against Ba or nicotine contractions. With strain III it was possible to reduce nicotine contractions with hexamethonium in dilutions of the order of 1 in a million, the activity relative to papaverine being 2.44 $\pm$ 2.15 (9 assays). Effects against Ba were, however, so variable that it was not possible to obtain an assay.

## Discussion

There are few records of substances many times more powerful than papaverine in reducing intestinal contractions due to barium. Jackman, Bolen, Nachod, Tullar, and Archer (1949) described 1-*cyclo*-pentylidene-3-dimethylamino-1-phenylpropane as 66 times more active than papaverine against barium spasm, and Becker, Ananenko, Glenwood, and Miller (1946) reported 1-*cyclo*-hexyl-1-phenyl-3-piperidinobutane to be 9 times as active as the reference drug. Few other substances have been described as more than 2 or 3 times as active as papaverine (see Bergel and Parkes, 1952). The order of activity found among the thioesters reported here is therefore outstanding and, indeed, suggests a specificity against barium corresponding to that of atropine against acetylcholine, or of such antihistamines as mepyramine against histamine. Only one or two of these compounds also possess strong activity against acetylcholine, of the order of that of atropine, and none is appreciably effective against histamine. High activity against barium in this series tends to be associated with high activity against potassium.

That the high anti-barium—and, to a lesser extent, anti-potassium—activity shown, on the guinea-pig ileum, by the compounds reported here may be due to a specific inhibitory action upon ganglion cells is supported by their even higher order of activity against nicotine, in the few instances in which this has been demonstrated. Further evidence is provided by the considerably lower order of activity against barium spasm in smooth muscle organs other than gut, in the responses of which ganglion-cell activity should play a smaller part. It has already been noted that ileum and tracheal chain preparations did not differ in sensitivity towards the inhibitory activity of Ro 3-0226 on acetylcholine, which Feldberg (1951) showed to be little affected by ganglion-blocking action in its effects upon intestine. The results also show that the thioesters are considerably weaker than papaverine in reducing the tone of tracheal muscle and coronary vessels, suggesting that papaverine-like activity in reducing smooth muscle tone plays little part in the strong spasmolytic activity that these substances exhibit on preparations of intestine.

Again, the highest order of activity against barium spasm was observed on the gut of the guinea-pig; the compounds were less effective on rabbit and rat gut. Feldberg (1951) showed that hexamethonium is less effective as a nicotine antagonist on rabbit than on guinea-pig intestine, and considered this difference to reflect the extent to which ganglion-cell activity is involved in the responses to nicotine in the two species. Similar conclusions could apply to the differences in responsiveness towards the compounds described here.

However, the differences between the activity of these compounds against barium and nicotine on the intestine of different strains of guinea-pig are more unexpected than those found between species. They may imply strain differences in the extent to which nervous elements are concerned in the response of the gut to barium and nicotine, or in the sensitivity of these elements towards inhibitors.

It has been remarked that the values given for relative activity against barium and nicotine are associated with a very high variance, frequently of the order of two-thirds of the mean value and rarely less than half. No amount of repetition has successfully reduced these differences, which occur whether many pieces of gut, from more than one animal, are used for only a single assay each or whether a number of assays are performed on a single piece of gut. The use of barium as a stimulant agent in tests for spasmolytic action has often been described as being associated with difficulties of this nature (see, for example, Lehmann and Knoefel, 1942) and here also the source of variability may lie in responsiveness of the nervous elements.

All these observations have implications for the question of papaverine-like spasmolytic activity. Papaverine is characterized by its power to reduce the activity of all smooth muscle preparations in similar effective concentrations, whatever the cause of activity, spontaneous or drug-induced—

a circumstance which justifies the description of its activity as "musculotropic." Early investigations employed responses to either histamine or barium in tests for such musculotropic inhibition, since these agents were considered to contract smooth muscle in a manner not as susceptible to specific antagonism as that of acetylcholine by atropine (see, for example, the review by Burtner, 1951). When specific inhibitors of histamine were found it was recognized that antagonism to this agent was not a suitable criterion of papaverine-like activity.

The evidence now presented suggests that antagonism to barium spasm may be as inappropriate as antagonism to histamine as a criterion of papaverine-like activity, since many of the compounds reported show an anti-barium activity many times that of papaverine on isolated guinea-pig ileum but little advantage over papaverine in reducing the activity of other forms of smooth muscle. The possibility of antagonism of the effect of barium upon gut by a mode of action different from that exerted by papaverine emphasizes that antagonism to barium should not necessarily be considered as evidence for papaverine-like properties. The unsuitability of this antagonism as the basis for a screening test is further demonstrated by the possibility of the dependence of relative activity upon the species, and even upon the strain, of animal used.

In consequence, it may be impossible to rely upon correspondence between results for anti-barium activity obtained by workers in different laboratories, or even in the same laboratory at different periods, unless it can be established that the same species, and also the same strain, of animal is used. Mention has already been made of the higher order of anti-barium activity found for diethylaminoethyl benzilate, Ro 3-0256, than that reported for this compound by Lehmann and Knoefel (1942) and Buchel *et al.* (1948). Results for some of the more active compounds shown in Table I demonstrate the variation in results at different periods, as work was not being restricted at this time to animals of one strain. It also seems likely that a relatively insensitive strain came into use at a certain point in the work, for high activities were noticeably absent among the compounds of higher serial numbers. The values for Ro 3-0367 (Table V) show that, although the original order of activity was not comparable with that of its homologues, that found later shows more correspondence. It is thus impossible to attempt to deduce any relationship between chemical structure and the values determined for anti-barium activity of the type found in this group.

## Summary

1. A number of compounds, principally amino-alkanethio- esters of benzilic acid, have been screened, on guinea-pig ileum, for spasmolytic activity against contractions produced by acetylcholine, histamine, barium and potassium chlorides, and nicotine. High activities against barium, potassium, and nicotine, of an order not previously reported, were found among this group.

2. The compounds were less active on the isolated intestine of the rabbit and the rat than on that of the guinea-pig. No compound was more active than papaverine on other forms of smooth muscle, such as uterus, bronchial muscle and coronary vessels. It is suggested that the mode of action of these inhibitors involves the nervous elements of the intestinal plexuses.

3. Great differences in activity against barium and nicotine on the intestine of several strains of guinea-pig are discussed.

4. The specificity of these compounds for antagonism of barium on the isolated intestine, particularly on the guinea-pig, probably indicates a different mode of action from that of papaverine.

5. It is concluded that anti-barium activity does not constitute a valid basis for testing synthetic compounds for papaverine-like spasmolytic activity.

I wish to thank Professor F. Bergel for much helpful encouragement and discussion during this work, and Dr. A. L. Morrison and the directors of Roche Products, Ltd., for permission to publish these results. I am indebted to Dr. D. F. Hawkins, who tested some of the compounds on the tracheal chain, and to Miss M. E. Cooke and Mr. N. D. Edge for technical assistance.

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# THE EFFECT OF COMPOUND 48/80 ON GANGLIONIC TRANSMISSION

BY

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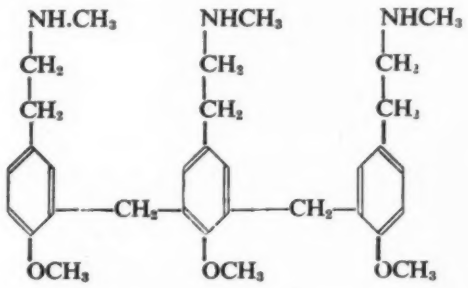
At the present time, compound 48/80 (a condensation product of *p*-methoxyphenethylmethylamine with formaldehyde), whose formula is given below (Baltzly, Buck, de Beer and Webb, 1949), is the most potent histamine liberator known (Paton, 1951 ; Feldberg and Paton, 1951). In addition, it has the ability to release heparin and a "slow contracting substance" from muscle and skin (Paton, 1951). It has now been demonstrated that 48/80 also has the property of blocking ganglionic transmission in the sympathetic ganglion of the cat. The site and mechanism of this block have been analysed.



48/80 is a mixture of dimer, trimer, and tetramer, with most of the pharmacological action residing in the trimer.

## METHODS

The experiments were carried out on the perfused superior cervical ganglion of the cat anaesthetized with chloralose (80 mg./kg.). The method of ganglionic perfusion was that described by Kibjakow (1933) and subsequently modified by Feldberg and Gaddum (1934) and by MacIntosh (see Perry, 1953). Locke's solution was used as the perfusion fluid (composition : NaCl 9.2 g., KCl 0.42 g., $CaCl_2$ 0.24 g., $NaHCO_3$ 0.15 g., dextrose 2.0 g., made up to 1 litre with distilled water).

The vagosympathetic trunk was cut peripherally and stimulated by means of square waves at a frequency of 10 cycles/sec., 0.5 msec. duration, and supermaximal voltage. The vagus was tied central to the ganglion, but, in order to diminish leakage of fluid during the perfusion, no separation of the peripheral vagosympathetic trunk was usually carried out. The ganglion with its preganglionic nerve was kept immersed in warm paraffin to prevent drying, and, by suspending a light bulb above the preparation, the temperature of the paraffin could be held between 36 and 37° C. As an indication of transmission in the ganglion, the contraction of the nictitating membrane was utilized and recorded on a smoked drum by a frontal writing lever. All drugs injected into the ganglion were dissolved in Locke's solution.

In several experiments the venous effluent from the perfused ganglion was assayed for histamine or ACh. When the ACh output was determined, the perfusion fluid contained eserine sulphate 1 in 100,000.

*Histamine Assay.*—Histamine in the effluent was assayed on the isolated atropinized guinea-pig ileum suspended in a 15 ml. bath in Mg-free Tyrode solution at 34° C. (composition : NaCl 8.0 g., KCl 0.2 g., $CaCl_2$ 0 2 g., $NaH_2PO_4$ 0.05 g., $NaHCO_3$ 1.0 g., dextrose 1.0 g., made up to 1 litre with distilled water). It was found in preliminary tests that 48/80 did not affect the response of the gut to histamine. If at all, there was a slight potentiation of response. All values of histamine are expressed as base.

*Acetylcholine Assay.*—When 48/80 and histamine were present in samples of the venous effluent, the perfusate could not be assayed for ACh on the arterial blood pressure of the cat because of the depressor action of 48/80. In preliminary tests it was found that histamine in a concentration of $10^{-6}$ and 48/80 in a concentration of $10^{-4}$ had no action on the eserinized dorsal muscle of the leech and did not influence the response of this muscle to ACh. The assay was therefore carried out on the leech muscle suspended at room temperature in a 5 ml. bath of a salt solution prepared by diluting the Locke's solution used for the ganglionic perfusion (1 litre Locke's solution to 1.4 litres with distilled water) and adding eserine sulphate 1 in 100,000. The routine adopted was to add the ACh or test solutions every 12 min. and allow the contraction to proceed for 2 min., thus

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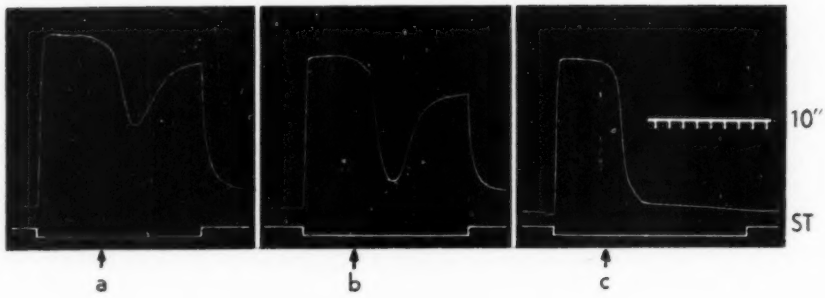


FIG. 1.—Perfused superior cervical ganglion of cat under chloralose anaesthesia. Contractions of nictitating membrane to stimulation of cervical sympathetic (ST). Injections into ganglion of 25 $\mu$g. 48/80 (at a), 50 $\mu$g. (at b), and 100 $\mu$g. (at c). Intervals of 10 min. between tracings. Time in 10 sec.

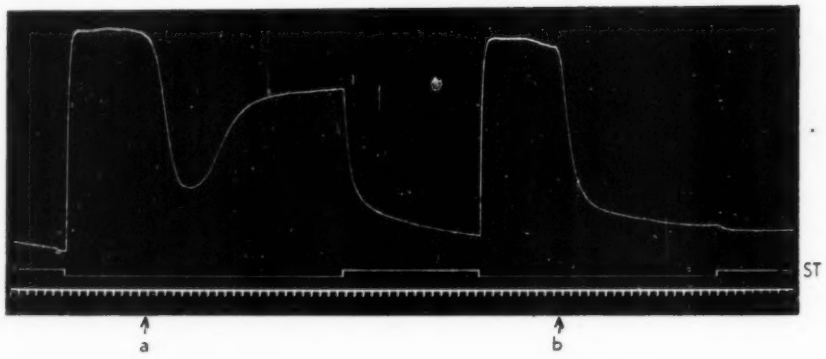


FIG. 2.—Perfused superior cervical ganglion of cat under chloralose anaesthesia. Contractions of nictitating membrane to stimulation of cervical sympathetic (ST). At a and b injection of 50 $\mu$g. 48/80 into ganglion. Time in 10 sec.

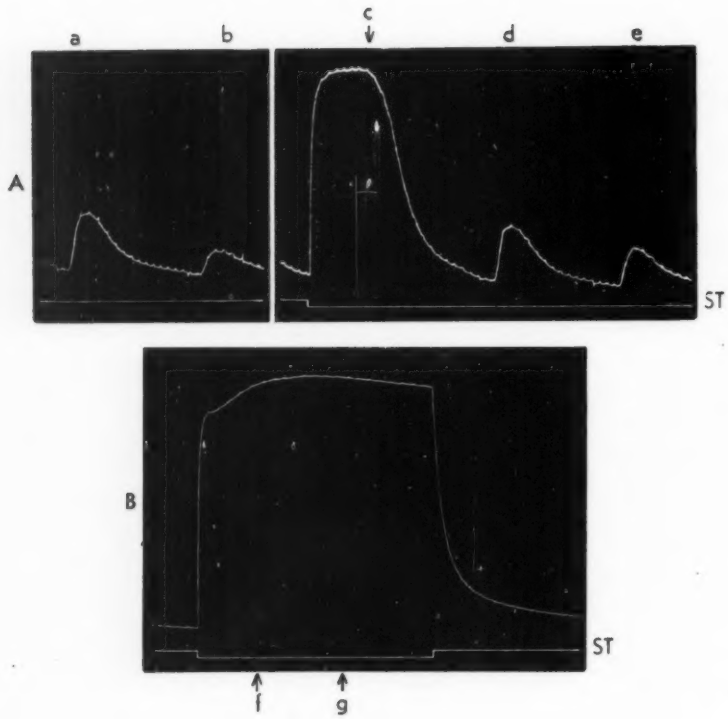


FIG. 3.—Perfused superior cervical ganglion of cat under chloralose anaesthesia. A, Contractions of nictitating membrane to intravenous administration of 15 $\mu$g. adrenaline at a, b, d, and e. At c, injection of 100 $\mu$g. 48/80 into ganglion during stimulation of cervical sympathetic (ST). B, Contraction of nictitating membrane to stimulation of postganglionic fibres (ST). Injections into ganglion of 100 $\mu$g. 48/80 (at f) and 10 mg. tetraethylammonium chloride (at g).

leaving a 10 min. interval between doses. This procedure yielded satisfactory assays, with the leech muscle remaining responsive for periods of over 6 hr.

## Results

### *Effects of 48/80 on Transmission*

48/80 had no stimulating action when injected into the ganglionic circulation in a dose of 25–100 μg. When injected during continuous maximal stimulation of the preganglionic fibres, however, it regularly produced an immediate relaxation of the nictitating membrane. The dose necessary to elict this effect varied in different experiments ; in some 25 μg. gave a pronounced action, in others only a slight relaxation or none at all. Fig. 1 illustrates a typical experiment. With 25 μg. 48/80 there was some relaxation of the nictitating membrane ; with 50 μg. the effect was greater, but complete relaxation was only obtained with 100 μg. With increasing doses of 48/80 the effect not only became more pronounced but also more prolonged. In the experiment of Fig. 1 the actions of 25 and 50 μg. 48/80 were over within 2 min., whereas the complete relaxation produced by 100 μg. persisted for about 10 min.

When a dose of 48/80 which caused partial relaxation of the nictitating membrane was repeated some minutes after complete recovery, it produced a greater and longer-lasting depression than the first. Fig. 2 illustrates this point.

The relaxation of the nictitating membrane is not due to escape into the blood stream of some of the injected 48/80 acting directly on the nictitating membrane, but is the result of a true ganglionic block. This is demonstrated by the fact that during the depression the nictitating membrane contracts both to adrenaline and to postganglionic stimulation, as illustrated in the experiments of Fig. 3. In Fig. 3 A are seen the contractions of the nictitating membrane produced by intravenous administration of 15 μg. adrenaline. At a and b the drug was injected before, and at d and e after, the administration of 100 μg. 48/80 (at c), during continuous stimulation of the cervical sympathetic. This dose of 48/80 produced complete relaxation of the nictitating membrane. In Fig. 3 B the contraction of the nictitating membrane produced by postganglionic stimulation is shown not to be affected by an injection into the ganglion of either 48/80 (at f) or the known ganglion blocking drug, tetraethylammonium (at g).

*Propamidine.*—A ganglionic blocking effect was also obtained with propamidine isethionate, another potent histamine liberator (MacIntosh and Paton, 1949). The doses required to elicit this action were of the same order as with 48/80.

### *Release of Acetylcholine During Ganglionic Block Produced by 48/80*

48/80 injected into the ganglionic circulation of an eserinized ganglion causes no output of ACh, nor does it affect the output of ACh during preganglionic stimulation, in spite of the occurrence of ganglionic block. This was shown by two series of experiments.

In the first, the preganglionic nerve was stimulated continuously and the perfusate collected every 2 min., the drug being injected on the second minute. During such continuous stimulation the ACh output is known to fall rapidly (Brown and Feldberg, 1936 ; Perry, 1953). This was confirmed, and is illustrated by the results of the first three experiments of Table I. The fall in ACh output was not modified by the administration of 48/80, as shown by the last three experiments of Table I. In these the injection of 100 μg. 48/80 caused pronounced block lasting for about the following 6 min. A comparison of the ACh outputs in these three experiments with the first three of Table I shows that the ganglionic block did not affect the release of ACh.

Table I

ACh OUTPUT (NG.) IN CONSECUTIVE 2 MIN. SAMPLES OF GANGLIONIC EFFLUENT DURING CONTINUOUS PREGANGLIONIC STIMULATION (10 CYCLES/SEC., 0.5 MSEC.)

Underlined values indicate ACh in sample collected during ganglionic block from injection of 100 μg. 48/80 into the ganglionic circulation

| Expt. No. | Consecutive Periods of Collection (min.) | | | | |
|---|---|---|---|---|---|
| | 0–2 | 2–4 | 4–6 | 6–8 | 8–10 |
| 1 | 33 | 30 | 25 | 22 | 20 |
| 2 | 35 | 30 | 21 | 21 | 19 |
| 3 | 62 | 39 | 20 | 12 | 10 |
| 4 | 34 | 27 | 20 | 15 | 10 |
| 5 | 47 | 35 | 28 | 18 | 12 |
| 6 | 36 | 30 | 25 | 20 | 15 |

In the second series of experiments, the preganglionic trunk was stimulated every 10 min. for 2 min. and the venous effluent collected during the 2 min. period of stimulation and the following 30 sec. This procedure has the advantage of excluding any possible carry-over of ACh from one period of stimulation to the next. As seen from a comparison of the first four experiments of Table II with the first three of Table I, the fall in ACh output during each subsequent 2 min. period of stimulation is approximately the same whether a period of rest is interpolated or not. In the last four experiments of Table II, 100 μg.

48/80 was injected 30 sec. before the second period of stimulation. The injection did not influence the release of ACh but caused profound block during the subsequent period of stimulation.

Since the first period of stimulation always yielded the greatest output of ACh, in one other experiment (No. 9 of Table II) 200 μg. 48/80 was injected 30 sec. before the first stimulation period, and blocked transmission completely so that there was no retraction of the nictitating membrane. Nevertheless, the output of ACh during this period was higher than that of the subsequent periods of stimulation, when the ganglionic block had worn off.

TABLE II

ACh OUTPUT (NG.) IN GANGLIONIC EFFLUENT FROM FIVE 2 MIN. PERIODS OF PREGANGLIONIC STIMULATION (AT 10 CYCLES/SEC., 0.5 MSEC. DURATION) WITH 8 MIN. REST BETWEEN EACH PERIOD

Underlined values indicate ACh in effluent collected during ganglionic block from injection of 100 μg. 48/80 into ganglionic circulation. Amounts injected in expts. 1–8 were 100 μg.; in expt. 9, 200 μg.

| Expt. No. | Collection Period | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 1 | 41 | 30 | 20 | 12 | 10 |
| 2 | 45 | 33 | 32 | 20 | 15 |
| 3 | 65 | 34 | 20 | 20 | 12 |
| 4 | 37 | 20 | 19 | 15 | 13 |
| 5 | 44 | 36 | 30 | 16 | 12 |
| 6 | 60 | 38 | 12 | 12 | 10 |
| 7 | 40 | 28 | 18 | 18 | 14 |
| 8 | 18 | 14 | 10 | 10 | 10 |
| 9 | 40 | 28 | 18 | 18 | 13 |

*Action of Acetylcholine During the Ganglionic Block Produced by 48/80*

During the ganglionic block produced by 48/80, the sensitivity of the ganglion cells to ACh is decreased. This is shown by the experiment of Fig. 4, in which the effects of 5 μg. ACh and of 10 sec. preganglionic stimulation were compared before (at a and b) and shortly after (at c and d) an injection of 75 μg. 48/80 into the ganglion. The effects of both ACh and of preganglionic stimulation were reduced approximately by half. Ten minutes later there was complete recovery for ACh as well as for preganglionic stimulation (at e and f). In other experiments of this kind complete recovery of the effect of preganglionic nerve stimulation sometimes preceded the recovery of response to injection of ACh by a few minutes.

The experiment of Fig. 5 A indicates that 48/80 not only affects the stimulating action of ACh on the ganglion but also the paralysing action, which effect has been attributed to an excess of ACh. At a, 5 μg. ACh was injected during preganglionic stimulation, producing a slight relaxation of the nictitating membrane. This relaxation suggests that the injected ACh, together with the released ACh, is sufficient to paralyse the ganglion. When the same dose of ACh was given shortly afterwards, during the block produced by 75 μg. 48/80, it had a stimulating action. As the release of ACh remains unaffected by 48/80, the sensitivity of the ganglion must have become reduced, so that a previously paralysing amount of ACh now exerted a stimulating action. The same result was obtained when the order was reversed. This is shown in Fig. 5 B, in which, instead of ACh, carbachol (carbaminoylcholine chloride) was injected during preganglionic stimulation—first during 48/80 block (at f) and later when the block had nearly worn off (at g). The paralysing effect (at g) was still preceded by a short-lasting stimulation.

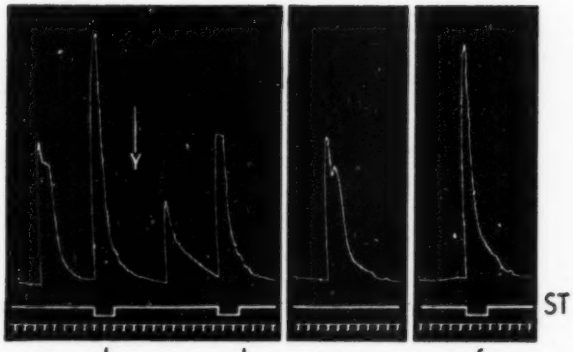


FIG. 4.—Perfused superior cervical ganglion of cat under chloralose anaesthesia. Contractions of nictitating membrane to injections into ganglion of 5 μg. ACh (at a, c, and e) and 10 sec. preganglionic stimulation (ST) at b, d, and f. At arrow, injection of 75 μg. 48/80 into ganglion. Between d and e, interval of 10 min. Time in 5 sec.

When 48/80 had completely blocked the effect of maximal preganglionic stimulation, an injection of ACh in larger doses still caused stimulation. Thus, 48/80 does not render the ganglion cells insensitive but only less sensitive to ACh.

*Histamine and Synaptic Transmission in the Sympathetic Ganglion*

Since 48/80 and propamidine are potent histamine liberators, the possibility had to be examined whether or not the block could be accounted for by the release of histamine. Kwiatkowski (1943) and von Euler (1949, 1950, 1951) have shown that many nerve fibres, particularly of the sympathetic system, contain rather large amounts of histamine, but it is not known if the histamine in nerves is susceptible to the action of histamine liberators.

When 48/80 was injected into the perfused ganglion, histamine appeared in the venous effluent.

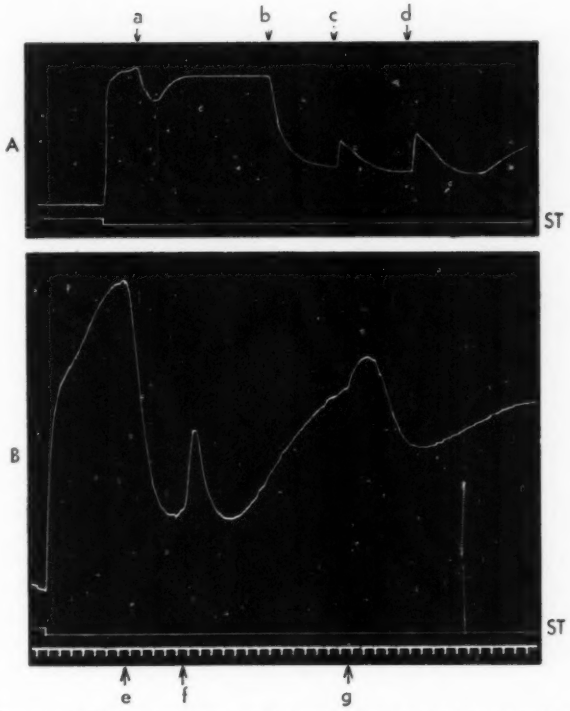


FIG. 5.—Perfused superior cervical ganglion of cat under chloralose anaesthesia. Contractions of nictitating membrane. A, Paralysing action of ACh converted by 48/80 into stimulating action. Stimulation of cervical sympathetic (ST). At a, c, and d, injection of 5 μg. ACh into ganglion; at b, 75 μg. 48/80. B, Partial conversion by 48/80 of stimulating action of carbachol into paralysing action. Stimulation of cervical sympathetic (ST). At f and g, injection of 5 μg. of carbachol; and at e, 75 μg. 48/80 into ganglion. Time in 10 sec.

The release of histamine, however, was apparently not responsible for the block because (1) histamine itself did not produce block, and (2) on repeated injections 48/80 still retained its blocking action when histamine release had ceased.

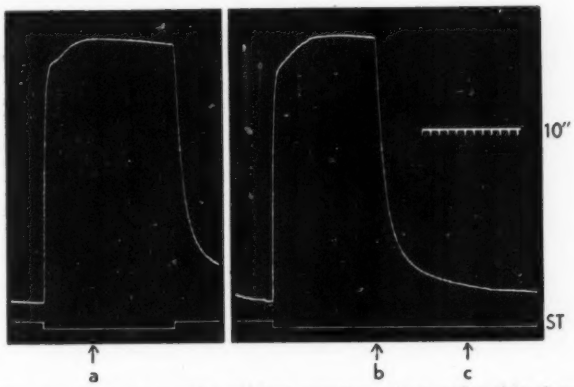


FIG. 6.—Perfused superior cervical ganglion of cat under chloralose anaesthesia. Contractions of nictitating membrane to stimulation of cervical sympathetic (ST). At a and c, injection of 100 μg. histamine into ganglion; at b, 100 μg. 48/80. Time in 10 sec.

*Effect of Histamine.*—The experiment of Fig. 6 shows that histamine injected during preganglionic nerve stimulation does not have any ganglionic blocking action and does not intensify the block produced by 48/80. In this experiment 100 μg. was injected at a, during maximal preganglionic stimulation, without producing any indication of even the smallest relaxation of the nictitating membrane. At c the same dose of histamine was injected during continuous preganglionic stimulation after block had been produced by 100 μg. of 48/80 (at b). The recovery from the block was not influenced by the histamine injection.

*Release of Histamine.*—An injection of 50 μg. 48/80 into the ganglion caused the appearance of histamine in the venous effluent; however, the amounts liberated varied greatly from animal to animal. The quantities released in seven experiments are shown in Table III, column 2; they varied between 0.05 and 0.9 μg. histamine. With repeated injections of the same dose of 48/80, the amounts of histamine liberated decreased from injection to injection until no histamine was detectable in the venous effluent with the method of assay employed. In the other experiments between 10 and 20% of the amounts of histamine released after the first injection were assayed in the perfusate collected after the second injection given 10 min. later. Once the injection of 48/80 had failed to liberate histamine, subsequent injections were likewise ineffective (see Table III, columns 3, 4, and 5). In two other experiments in which the ganglion was stimulated during the time 48/80 was injected, similar outputs of histamine to those reported in Table III were obtained. In all of the experiments reported here, mepyramine maleate could be shown to block the histamine response of the guinea-pig ileum, indicating that the active principle was in fact histamine.

TABLE III

HISTAMINE RELEASED FROM THE UNSTIMULATED PERFUSED SUPERIOR CERVICAL GANGLION BY REPEATED INJECTIONS OF 50 μG. 48/80 AT 10 MIN. INTERVALS

| Expt. No. | Amount of Histamine (μg.) | | | |
|---|---|---|---|---|
| | 1st Inj. | 2nd Inj. | 3rd Inj. | 4th Inj. |
| 1 | 0·05 | 0·005 | 0·005 | — |
| 2 | 0·12 | — | — | — |
| 3 | 0·07 | 0·03 | — | — |
| 4 | 0·20 | 0·04 | 0·01 | 0·005 |
| 5 | 0·09 | 0·005 | — | — |
| 6 | 0·90 | 0·02 | 0·005 | — |
| 7 | 0·08 | — | — | — |

## Discussion

The histamine liberators, 48/80 and propamidine, have at least one other action in common which has hitherto not been described—that of producing ganglionic block in the perfused sympathetic ganglion. Analysis of the block produced by 48/80 has shown that the release of ACh from the preganglionic endings is not interfered with, but that the sensitivity of the ganglion cells to the action of ACh is reduced. There was no evidence that the block could be explained by persistent depolarization, since neither 48/80 nor propamidine excited the ganglion cells to discharge before the onset of block. Furthermore, it was possible to overcome the block, at least partially, by injecting a large dose of ACh into the perfused ganglion.

Histamine is a normal constituent of nervous tissue. It was therefore natural to consider the possibility that the histamine liberators produce the block by the release of histamine from the ganglion, particularly when it was found that 48/80 did release histamine from the perfused ganglion. Nevertheless, this possibility could be excluded because of the following three facts: (1) Injections of histamine into the ganglion do not impair ganglionic transmission. A similar observation was made by Feldberg and Vartiainen (1934). (2) The block produced by 48/80 is not enhanced by a subsequent injection of histamine. (3) The block produced by 48/80 bears no relation to the amounts of histamine released. These decrease with repeated injections of the same dose of 48/80, whereas the block becomes more pronounced with each injection. After a few injections, when histamine release can no longer be detected, 48/80 exerts an even stronger blocking action than after the first injection.

Nevertheless, we must be cautious at the present stage of our knowledge in excluding any connexion between histamine liberating and ganglion blocking properties of 48/80 and propamidine. We do not know if there are histamine liberators which lack the ability to produce ganglionic block. Certainly one other known, but less potent, histamine liberator is also a ganglion blocking substance—namely, (+)-tubocurarine. On the other hand, there are many ganglion blocking drugs which are not histamine liberators.

That 48/80 and propamidine both block the transmission of impulses across the sympathetic ganglion is of particular interest in relation to some of those systemic effects of histamine liberators which are not readily explicable by an action of released histamine. For example, Nasmyth (1955) has recently shown that, in rats depleted of their tissue histamine by repeated injections of 48/80, this compound still produces a fall in arterial blood pressure, although the depressor effect is much milder than in normal rats. He discusses the problem of whether this reduced effect is due to release of small residual amounts of tissue histamine or to an unspecific action of 48/80. The ganglion blocking effect of 48/80 could easily explain this finding.

It was found that in the perfused ganglion histamine was ineffective not only in impairing ganglionic transmission but also in stimulating the ganglion. Trendelenburg (1954) has observed a ganglion stimulating action with histamine, but his experiments were performed in a ganglion with its normal blood circulation intact. It is thus possible that any ganglion stimulating action of histamine is lost when the ganglion is perfused with artificial salt solution.

48/80, a secondary amine, and propamidine, a diguanidine, do not bear any great chemical similarity to onium compounds, such as hexamethonium and the thiophanium drugs, which possess such strong ganglion blocking actions. From a purely structural comparison, there seems to be little basis for explaining why propamidine and 48/80 block ganglionic transmission, whereas histamine, another amine derivative, exerts no such action at all. If the ganglionic blocking effects were purely a non-specific action of amines or guanidines in general, one would expect them all, including histamine, to exert ganglionic actions.

48/80 is chemically very similar to the sympathomimetic amines, some of which—adrenaline and ephedrine, for example—have been shown to depress ganglionic transmission (Bülbring and Burn, 1942; Marrazzi, 1939). As yet no typical sympathomimetic actions have been described for 48/80, possibly because of the histamine release obscuring any other pharmacological actions. If such effects, however weak, are reported in the future, one would then have to consider the possibility that 48/80 owed its ganglionic blocking effect to its structural resemblance to sympathomimetic amines.

## Summary

1. 48/80 and propamidine cause ganglionic block in the perfused superior cervical ganglion of the cat. This block has been analysed for 48/80.

2. 48/80 does not produce block by decreasing the amount of acetylcholine released from the preganglionic endings, but by rendering the ganglion cells less sensitive to the released ACh.

3. 48/80 releases histamine from the perfused ganglion.

4. The release of histamine is not the cause of block produced by 48/80, because: (*a*) histamine does not impair ganglionic transmission; (*b*) the block produced by 48/80 is not enhanced by histamine; (*c*) the amounts of histamine released decrease, whereas the block becomes stronger with each injection of 48/80. When 48/80 no longer releases histamine, it still exerts its blocking action.

I am most indebted to Dr. W. Feldberg for his valuable advice during the course of this investigation.

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# THE EFFECTS OF SODIUM SALICYLATE ON BLOOD GLUCOSE IN THE RAT

BY

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The injection of salicylate into normal male rats has been observed to cause a marked depletion of the liver glycogen level at 4 hr., but the blood glucose concentration remained constant (Lutwak-Mann, 1942; Smith, Meade, and Bornstein, 1952). A similar reduction in the liver glycogen content of mice 1 and 2 hr. after the injection of salicylate was reported by Sproull (1954). No significant alteration in the blood glucose level accompanied this change in the liver glycogen in male mice, but female mice showed a hyperglycaemia.

The effect of salicylate on the blood glucose of male and female rats has therefore been studied to determine if this sex difference also applied to rats. The effect of fasting for 24 hr. before the experiment was also investigated, and, in order to assess the influence of the adrenal, similar experiments were performed on adrenal-demedullated and on adrenalectomized rats.

## METHODS

*Animals.*—Rats of the Wistar strain, weighing 200–300 g. and maintained on a diet of cubes (M.R.C. Diet 41), were used. Adrenal demedullation was performed according to the directions in Farris and Griffith (1949). The rats were given 0.45% (w/v) NaCl soln. to drink instead of water for the first post-operative week only, and were used 40 days after operation. The bilaterally adrenalectomized rats were used eight days after operation, having been maintained on 0.45% (w/v) NaCl soln. instead of drinking water. The animals were used in groups of 20 which were divided into two sub-groups. One sub-group received an intraperitoneal injection of 0.5 mg./g. body weight of a 10% (w/v) solution of sodium salicylate and the other was given a similar injection of an equal volume of 0.9% (w/v) NaCl soln. Blood glucose estimations by the method of Nelson (1944) were made on tail-vein samples obtained immediately before and at intervals of 1, 2, 3, and 4 hr. after the injections. The rats were either deprived of food only from the beginning of the experiment (non-fasted animals) or for 24 hr. previously (fasted animals).

The following groups of rats were used: (1) non-fasted and fasted females; (2) non-fasted and fasted males; (3) non-fasted and fasted adrenal-demedullated males; and (4) non-fasted adrenalectomized males.

## RESULTS

The blood glucose results on female rats are given in Table I. In all the following tables the significances of the differences between the means have been analysed by Student's *t*-test, and values of P are included. The minimum acceptable level of significance has been taken as $P=0.02$.

TABLE I

THE EFFECT OF SALICYLATE ON THE BLOOD GLUCOSE OF NON-FASTED AND FASTED FEMALE RATS

Mean values ± S.E. (no. of rats in parentheses) for blood glucose levels expressed as mg./100 ml.

| Time after Injection (hr.) | Non-fasted | | | Fasted | | |
|---|---|---|---|---|---|---|
| | Control (8) | Salicylate-treated (12) | P | Control (8) | Salicylate-treated (11) | P |
| 0 | 88·6 ± 4·5 | 83·6 ± 9·0 | 0·1 | 70·6 ± 7·0 | 64·8 ± 5·4 | 0·1 |
| 1 | 93·6 ± 6·0 | 129·1 ± 10·6 | 0·001 | 69·3 ± 7·5 | 81·5 ± 10·0 | 0·02 |
| 2 | 91·7 ± 6·0 | 118·5 ± 15·6 | 0·001 | 78·5 ± 7·5 | 98·6 ± 11·5 | 0·001 |
| 3 | 90·6 ± 7·5 | 105·0 ± 8·4 | 0·01 | 75·0 ± 11·1 | 90·5 ± 13·2 | 0·02 |
| 4 | 90·1 ± 6·2 | 91·6 ± 11·7 | 0·8 | 73·3 ± 5·9 | 87·0 ± 17·9 | 0·05 |

The results show that the injection of sodium salicylate caused a significant but moderate hyperglycaemia in both non-fasted and fasted female rats 1, 2, and 3 hr. after the injection. The effect appeared to be more prolonged in the fasted animals, although the results of the salicylate-treated and control animals at 4 hr. were not significantly different.

The blood glucose results on male rats are given in Table II.

The results are essentially similar to those obtained with the female rats: a hyperglycaemia occurred 1 and 2 hr. after the injection in the non-fasted males and persisted for 4 hr. in the fasted male animals.

TABLE II

THE EFFECT OF SALICYLATE ON THE BLOOD GLUCOSE OF NON-FASTED AND FASTED MALE RATS

Mean values±S.E. (no. of rats in parentheses) for blood glucose levels expressed as mg./100 ml.

| Time after Injection (hr.) | Non-fasted | | | Fasted | | |
|---|---|---|---|---|---|---|
| | Control (8) | Salicylate-treated (12) | P | Control (6) | Salicylate-treated (11) | P |
| 0 | 87·1±7·0 | 81·4± 6·3 | 0·1 | 68·0±3·3 | 67·4± 5·7 | 0·9 |
| 1 | 90·4±5·2 | 127·9±14·2 | 0·001 | 69·3±6·2 | 87·9±13·4 | 0·01 |
| 2 | 90·5±6·6 | 118·9±17·7 | 0·001 | 68·0±5·6 | 91·5±13·5 | 0·01 |
| 3 | 90·4±7·2 | 95·0±12·1 | 0·4 | 75·0±9·3 | 93·5±13·3 | 0·02 |
| 4 | 83·8±6·6 | 83·5± 9·7 | 0·9 | 77·3±8·7 | 94·5± 9·1 | 0·01 |

TABLE III

THE EFFECT OF SALICYLATE ON THE BLOOD GLUCOSE OF NON-FASTED AND FASTED MALE ADRENAL-DEMEDULLATED RATS

Mean values±S.E. (no. of rats in parentheses) for blood glucose levels expressed as mg./100 ml.

| Time after Injection (hr.) | Non-fasted | | | Fasted | | |
|---|---|---|---|---|---|---|
| | Control (8) | Salicylate-treated (10) | P | Control (8) | Salicylate-treated (11) | P |
| 0 | 84·2±8·0 | 78·1±14·9 | 0·4 | 73·1±12·1 | 66·0±6·2 | 0·1 |
| 1 | 85·0±6·2 | 101·3±15·9 | 0·02 | 71·1± 5·4 | 74·1±8·3 | 0·4 |
| 2 | 88·5±9·1 | 82·9±17·4 | 0·5 | 76·5± 6·3 | 82·0±7·3 | 0·2 |
| 3 | 89·9±7·3 | 78·1±11·4 | 0·05 | 74·9± 4·2 | 78·3±5·3 | 0·5 |
| 4 | 90·5±6·8 | 76·9±16·2 | 0·05 | 74·4± 7·1 | 77·2±6·2 | 0·4 |

The results on the male adrenal-demedullated rats are given in Table III.

In the non-fasted animals a significant difference was only obtained 1 hr. after the injection ; this effect was not observed in the fasted animals.

Table IV gives the results on the non-fasted male adrenalectomized rats.

The injection of salicylate produced an immediate and rapid fall in the blood glucose. This usually proved fatal, 10 of the salicylate-treated animals dying with hypoglycaemic convulsions within 4 hr.

TABLE IV

EFFECT OF SALICYLATE ON THE BLOOD GLUCOSE OF NON-FASTED MALE ADRENALECTOMIZED RATS

Mean values±S.E. (no. of rats in parentheses) for blood glucose levels expressed as mg./100 ml.

| Time after Injection (hr.) | Control | Salicylate-treated | P |
|---|---|---|---|
| 0 | 98·0±11·4 (8) | 99·1± 6·9 (12) | 0·7 |
| 1 | 88·0±13·9 (8) | 71·4± 8·2 (11) | 0·01 |
| 2 | 82·0±14·1 (8) | 51·0±10·7 (8) | 0·001 |
| 3 | 72·0±13·3 (8) | 43·5±10·9 (6) | 0·01 |
| 4 | 70·7±14·0 (7) | 43·0± 3·5 (2) | 0·05 |

## DISCUSSION

The present work was designed primarily to determine if there is a sex difference in the response of the blood glucose concentration to salicylate in the rat. It was observed that both male and female rats, whether non-fasted or fasted previously to the experiment, showed hyperglycaemia after the injection of salicylate. The rat does not therefore show a sex difference to salicylate similar to that reported for the mouse (Sproull, 1954). In the non-fasted animals the hyperglycaemia did not persist beyond 3 hr. ; the present results do not conflict with the observation (Lutwak-Mann, 1942 ; Smith *et al.*, 1952) that there was no change in the blood glucose concentrations of non-fasted male rats 4 hr. after the injection of salicylate.

The hyperglycaemia produced by the salicylate may have been due to one or more causes, such as increased liver glycogenolysis, an increased intestinal absorption of glucose, a decreased tissue utilization of glucose and increased gluconeogenesis. Since it was known that salicylate produced a marked depletion of liver glycogen in the rat (Lutwak-Mann, 1942) the possibility was considered that the hyperglycaemia may have been secondary to increased hepatic glycogenolysis. Comparison of the results from the non-fasted and fasted rats (Tables I and II) showed that the hyperglycaemia was more pronounced in the former animals, which may be a reflection of their higher content of liver glycogen. Although the liver glycogen content of fasted rats is relatively low these animals showed a definite hyperglycaemic response to salicylate which appeared to persist for a longer period than in the non-fasted animals. Fasted animals show impaired glucose tolerance curves and are not able to utilize administered carbohydrate as rapidly as the fed animal (Chambers, 1938 ; Lundbaek, 1948), so that the glucose produced by increased hepatic glycogenolysis in the fasted salicylate-treated rat may not have been removed as rapidly as in the fed rat.

A further difference is the presence of food in the gut of the fed animals ; but it has been shown (Smith and Irving, 1955) that salicylate causes a marked delay in the rate of gastric emptying in the fed rat, which is therefore similar to the fasted salicylate-treated animal, in that although food is present in the stomach it is not available for digestion and absorption for at least several hours.

If the hyperglycaemia produced by salicylate was secondary to increased hepatic glycogenolysis it could have been mediated by an increased

release of adrenaline from the adrenal medulla. The fasted adrenal-demedullated rat did not exhibit a hyperglycaemia when treated with salicylate, and the hyperglycaemic response was greatly reduced in the non-fasted salicylate-treated adrenal-demedullated rat. These results suggest that the secretion of adrenaline from the adrenal medulla played an important part in the production of the hyperglycaemic response to salicylate in the rat. However, the presence of a significant degree of hyperglycaemia 1 hr. after the injection of salicylate in the non-fasted adrenal-demedullated animal means that salicylate can also produce an increased blood glucose by some other mechanism. One possible explanation is that salicylate acts directly on the liver, causing increased breakdown of glycogen: it has been found that incubation of liver slices with salicylate *in vitro* causes an increased rate of disappearance of glycogen (Smith, 1955).

Most non-fasted adrenalectomized rats given salicylate died with hypoglycaemic convulsions within 4 hr. of the injection. This hypoglycaemic effect resembles the marked reduction in blood glucose produced by salicylate in the alloxan-diabetic rat (Smith *et al.*, 1952). It has been suggested that salicylate causes an increased rate of tissue glycolysis by interfering with oxidative phosphorylation processes (Smith, 1954).

It is suggested, as a provisional hypothesis, that the injection of salicylate to a normal rat causes an increased secretion of adrenaline from the adrenal medulla. The adrenaline produces increased hepatic glycogenolysis, and the hyperglycaemia is a secondary phenomenon. This is not considered to be a specific effect of salicylate but merely a non-specific stimulation of the adrenal medulla due to a relatively large dose of the drug. There may also be a direct action of salicylate on liver glycogen because of the 1-hr. hyperglycaemia observed in the non-fasted adrenal-demedullated rat. The hypoglycaemic effect of salicylate in the adrenalectomized rat emphasizes the importance of the adrenal cortex in maintaining the blood glucose concentration, and may be due to a more fundamental and specific action of salicylate on carbohydrate metabolism.

## Summary

1. The blood glucose concentrations of rats were determined 1, 2, 3, and 4 hr. after the injection of sodium salicylate, 0.5 mg./g. body weight.

2. Moderate hyperglycaemia was observed in normal rats, irrespective of sex, non-fasted and fasted alike, at 1, 2, and 3 hr. following the administration of salicylate; at 4 hr. the blood glucose level returned to normal in females, but remained slightly elevated in fasted males.

3. Hyperglycaemia was less pronounced in adrenal-demedullated rats treated with salicylate; in adrenalectomized rats salicylate caused a marked decrease in blood glucose concentration.

4. Some possible mechanisms are discussed.

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# THE THERAPEUTIC ACTIVITY OF SOME SULPHONES AND SULPHOXIDES IN EXPERIMENTAL TUBERCULOSIS OF GUINEA-PIGS

BY

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It has been suggested by Anand, Vyas, and Dhar (1953) that a lipophilic drug would have a good chance of reaching lipid-rich mycobacteria and the harbouring tissues. Based on this assumption, a series of unsymmetric sulphides, sulphoxides, and sulphones carrying an alkylamino-group at one end and a free or potentially free amino-group at the other end of the molecule have been synthesized (Anand *et al.*, 1953 ; Anand, Wadia, and Dhar, 1954 ; Khosla, Anand, and Dhar, 1954 ; Vyas, Anand, and Dhar, 1954).

Some of these compounds have been tested for their therapeutic value in experimental tuberculosis of guinea-pigs and the results are presented in this paper.

## MATERIALS AND METHODS

The drugs used were: SN 44 (*p*-ethylamino-*p'*-aminodiphenyl sulphone) ; SN 45 (*p*-propylamino-*p'*-aminodiphenyl sulphone) ; SN 47 (*p-iso*butylamino-*p'*-aminodiphenyl sulphone) ; SN 50 (*p-iso*amylamino-*p'*-aminodiphenyl sulphone) ; SN 89 (*p*-methylamino-*p'*-aminodiphenyl sulphoxide) ; SN 105 (Na salt) (*p*-methylamino-*p'*-sodium formaldehyde sulphoxylate aminodiphenyl sulphone) ; SN 153 (*p*-(ω-iodoethylamino)-*p'*-aminodiphenyl sulphone) ; SN 199 (*p*-ethylamino-*p'*-amino-*o*-hydoxyphenyl sulphone).

In the first experiment the therapeutic activity of SN 44, SN 45, SN 50, and SN 89 was compared with dihydrostreptomycin sulphate (DHS). Thirty-six tuberculin-negative guinea-pigs of about 300 g. in weight were inoculated subcutaneously in the right groin with 2 mg. (moist weight) of an 11-day culture of *Mycobacterium tuberculosis var. hominis* strain H 37 Rv, in Lowenstein-Jensen medium, suspended in 0.5 ml. of basal Dubos medium containing Tween 80. The animals were divided into groups of six each. The first group served as the untreated control, while groups two to six were treated with DHS, SN 44, SN 45, SN 50, and SN 89 respectively. DHS was administered subcutaneously, whereas the other drugs were suspended in water with 1% tragacanth and fed orally through a syringe. The treatment was begun on the tenth day and was continued daily for a period of 54 days. The average daily dose of the drugs in g./kg. of body weight is given in Table I. All the surviving animals were sacrificed at the end and examined post-mortem ; the animals which died during the experiment were also examined. A few guinea-pigs which died of causes other than tuberculosis were left out of consideration.

In a second experiment the activity of drugs SN 44, SN 47, SN 153, SN 199, SN 105 (Na salt), and SN 89 was compared with that of isonicotinic acid hydrazide (isoniazid, INH) and *p-p'*-diaminodiphenyl sulphone (dapsone, DDS). Fifty tuberculin-negative guinea-pigs were infected in the manner described above, using the same strain. The infecting dose was reduced to 0.5 mg. (moist weight) in order to produce a less severe disease. All the animals were seen to react to old tuberculin (Burroughs Wellcome) on the 20th day. On the 22nd day five animals were necropsied, and macroscopic lesions were seen at the site of inoculation, inguinal glands, spleen, and liver. The remaining animals were then divided into 9 groups of 5 animals each. The first group was kept as untreated control, while the rest were fed with drugs as before. Treatment was commenced on the 22nd day and was continued for 55 days. The average daily dose of the drugs in g./kg. of body weight is given in Table III.

*Necropsy Score.*—The following scores were given on post-mortem examination: 0, no lesions; 1, slight lesions (miliary lesions with no necrosis) ; 2, moderate lesions (nodular tubercles with slight necrosis) ; 3 to 4, severe lesions (moderate to severe necrosis).

*Histopathology.*—Sections of the lung, liver, spleen, and local glands, after fixing and staining, were studied for the presence of "active" lesions (necrotic foci) or "inactive" lesions (fibrosis, calcification, or epithelioid cell collection with no necrosis). The percentage of "active" lesions was calculated on the basis of the number of tissues showing these lesions. Since only four tissues (lung, liver, spleen, and gland) were examined, the total number of tissues was obtained by multiplying the number of animals by four. The percentage of "inactive" and of "no"

lesions were calculated in a similar manner (Karlson and Feldman, 1953).

Tuberculin tests and spleen cultures were made on all animals employed in the second experiment at the conclusion of the test.

## Results

None of the drugs employed in this investigation produced any toxic symptoms in the animals, and the tissues of the lung, liver, spleen, and kidney did not show any damage attributable to the drug. In the first experiment SN 44 and DHS were seen to be therapeutically more active than SN 45, SN 50, and SN 89, judged from the percentage of infectivity (Table I). "Active" lesions were least in animals treated with these two drugs (Table II) and were confined to the local glands. There was a corresponding preponderance of animals with "no" lesions. Active lesions were maximal in the untreated animals, whereas animals treated with SN 45, SN 50, and SN 89 occupied an intermediate position.

In the second experiment SN 44 and SN 47 were as good as INH. SN 153 and DDS came next in the order of therapeutic activity. SN 199 and SN 89 were less effective than the rest, while SN 105 (Na salt) was ineffective. The percentage infectivity data is presented in Table III. Histopathological findings agreed in general with the necropsy findings. "Active" lesions were least in animals treated with SN 44 and SN 47, while a high percentage of "no" lesions was seen in animals treated with SN 44 and INH (Table IV). Spleen cultures from animals treated with SN 44, SN 47, SN 153, DDS and INH proved negative for *M. tuberculosis*. On the contrary *M. tuberculosis* could be recovered from the spleens of animals treated with SN 199, SN 89, and SN 105 (Na salt).

Tuberculin tests carried out after 55 days of treatment showed a diminution in the intensity of reaction in animals treated with INH, SN 44, SN 47, and DDS as compared with the control animals. There was no necrosis and the oedema was appreciably less. Two animals of the SN 44 group and one animal of the SN 47 group gave a doubtful reaction of erythema without oedema.

TABLE I

THERAPEUTIC ACTIVITY OF SULPHONES AND SULPHOXIDES COMPARED WITH DIHYDROSTREPTOMYCIN SULPHATE IN EXPERIMENTAL TUBERCULOSIS

| Drug | No. of Animals | Average Daily Dose g./kg. | Average Post-mortem Scores of Infectivity* | | | | | %† Infection |
|---|---|---|---|---|---|---|---|---|
| | | | Lung | Liver | Spleen | Local Gland | Total (Max. =12) | |
| Control (no drug) | 6 | Nil | 3·0 | 2·5 | 2·8 | 3·0 | 11·3 | 94·2 |
| DHS .. | 4 | 0·03 | 0 | 0 | 0·7 | 1·5 | 2·2 | 18·7 |
| SN 44 .. | 5 | 0·045 | 0 | 0 | 0 | 2·3 | 2·3 | 19·4 |
| SN 45 .. | 6 | 0·03 | 0·8 | 0·8 | 1·5 | 3·0 | 6·2 | 51·3 |
| SN 50 .. | 6 | 0·03 | 0 | 2·0 | 1·5 | 3·0 | 6·5 | 54·1 |
| SN 89 .. | 6 | 0·03 | 0·2 | 2·4 | 1·6 | 2·6 | 6·8 | 56·7 |

* Score of infectivity: 0 (normal) to 3 (severe).

† % infection = $\frac{\text{Actual total score}}{\text{Maximum total score}} \times 100$.

TABLE II

EFFECT OF SULPHONES AND SULPHOXIDES COMPARED WITH DIHYDROSTREPTOMYCIN SULPHATE (DHS) ON TISSUES OF INFECTED GUINEA-PIGS AS REVEALED BY MICROSCOPIC EXAMINATION

| Drug | No. of Animals | Total No. of Tissues | Active Lesions | | Inactive Lesions | | No Lesions | |
|---|---|---|---|---|---|---|---|---|
| | | | No. | % | No. | % | No. | % |
| Control (no drug) | 6 | 24 | 21 | 87·5 | 2 | 8·3 | 1 | 4·1 |
| DHS .. | 4 | 16 | 4 | 25·0 | 1 | 6·2 | 11 | 68·7 |
| SN 44 .. | 5 | 20 | 5 | 25·0 | 3 | 15·0 | 12 | 60·0 |
| SN 45 .. | 6 | 24 | 10 | 41·7 | 11 | 45·8 | 3 | 12·5 |
| SN 50 .. | 6 | 24 | 12 | 50·0 | 8 | 33·3 | 4 | 16·7 |
| SN 89 .. | 6 | 24 | 17 | 70·8 | 6 | 25·0 | 1 | 4·2 |

TABLE III

ACTIVITY OF SULPHONES AND SULPHOXIDES COMPARED WITH ISONIAZID (INH) AND DAPSONE (DDS) IN EXPERIMENTAL TUBERCULOSIS OF GUINEA-PIGS

| Drug | No. of Animals | Average Daily Dose g./kg. | Average Post-mortem Scores of Infectivity* | | | | | %† Infection |
|---|---|---|---|---|---|---|---|---|
| | | | Lung | Liver | Spleen | Local Gland | Total (Max. =16) | |
| Control (no drug) | 5 | Nil | 3·5 | 3·5 | 3·1 | 3·5 | 13·6 | 85·0 |
| DDS .. | 5 | 0·1 | 0·3 | 1·7 | 1·0 | 2·7 | 5·7 | 35·6 |
| INH .. | 5 | 0·015 | 0 | 0 | 2·0 | 2·5 | 4·5 | 28·1 |
| SN 44 .. | 5 | 0·1 | 0·2 | 0·2 | 0·7 | 2·0 | 3·2 | 20·3 |
| SN 47 .. | 5 | 0·1 | 0 | 0·5 | 1·3 | 2·0 | 3·8 | 23·7 |
| SN 153 | 5 | 0·1 | 0·6 | 1·6 | 1·6 | 2·4 | 6·2 | 38·7 |
| SN 199 | 5 | 0·1 | 1·0 | 2·6 | 2·0 | 3·0 | 8·6 | 53·7 |
| SN 89 .. | 5 | 0·1 | 1·0 | 2·5 | 1·5 | 3·5 | 8·5 | 53·1 |
| SN 105 (Na salt) | 5 | 0·1 | 3·0 | 2·8 | 3·3 | 3·5 | 12·6 | 78·7 |

* Score of infectivity: 0 (normal) to 4 (severe).

† % infection = $\frac{\text{Actual total score}}{\text{Maximum total score}} \times 100$.

TABLE IV

EFFECT OF SULPHONES AND SULPHOXIDES COMPARED WITH ISONIAZID (INH) AND DAPSONE (DDS) ON TISSUES OF INFECTED GUINEA-PIGS AS REVEALED BY MICROSCOPIC EXAMINATION

| Drug | No. of Animals | Total No. of Tissues | Active Lesions | | Inactive Lesions | | No Lesions | |
|---|---|---|---|---|---|---|---|---|
| | | | No. | % | No. | % | No. | % |
| Control (no drug) | 5 | 20 | 18 | 90·0 | 2 | 10·0 | 0 | 0 |
| INH .. | 5 | 20 | 7 | 35·0 | 1 | 5·0 | 12 | 60·0 |
| DDS .. | 5 | 20 | 8 | 40·0 | 9 | 45·0 | 3 | 15·0 |
| SN 44 .. | 5 | 20 | 4 | 20·0 | 4 | 20·0 | 12 | 60·0 |
| SN 47 .. | 5 | 20 | 3 | 15·0 | 8 | 40·0 | 9 | 45·0 |
| SN 153 | 5 | 20 | 6 | 30·0 | 10 | 50·0 | 4 | 20·0 |

Based on these two experiments it seems safe to conclude that SN 44 and SN 47 were as good as INH and DHS in the treatment of experimental tuberculosis in guinea-pigs. Promin, diasone, promizole, solapsone and other derivatives of diaminodiphenyl sulphone are known to act synergistically or additively with streptomycin in the treatment of experimental as well as human tuberculosis (references in Kolmer, 1951). It may therefore be of interest to study the combined action of these lipophilic sulphones, SN 44 and SN 47, with streptomycin or isoniazid. The sulphones now in the therapeutic field are less lipophilic than those employed in this investigation.

## Summary

Of some sulphones and sulphoxides tested for therapeutic activity in experimental tuberculosis of guinea-pigs, SN 44 (*p*-ethylamino-*p'*-aminodiphenyl sulphone) and SN 47 (*p-iso*butylamino-*p'*-aminodiphenyl sulphone) were found to be active and to be comparable with dihydrostreptomycin sulphate and isonicotinic acid hydrazide.

The authors are grateful to Mr. I. S. Mathur for his technical assistance. They also wish to thank Dr. B. N. Singh, Assistant Director (Microbiology Division), for his help and guidance.

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# OBSERVATIONS ON Ω-AMINO-POLYMETHYLENE TRIMETHYLAMMONIUM COMPOUNDS

BY

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The series of bis-onium compounds which includes hexamethonium and decamethonium (Barlow and Ing, 1948; Paton and Zaimis, 1949) has been extensively studied. The high biological activity of the members of this series is closely linked with the presence, in one molecule, of the two onium groups. If these groups are replaced by amino groups, as in hexamethylene diamine or decamethylene diamine, compounds result which are without the characteristic biological action of the onium compound.

As far as is known the bis-onium compounds are not metabolized in the animal body. Zaimis (1950) has shown that hexamethonium is excreted as such in the urine. On the other hand, the diamines are broken down. This was first shown for tetramethylene diamine and pentamethylene diamine by Udranszky and Baumann (1891). More recently, the oxidation of the members of the homologous series $H_3\overset{+}{N}(CH_2)_n\overset{+}{N}H_3$ by the enzymes amine oxidase and histaminase has been studied (Blaschko and Hawkins, 1950). It was found, in agreement with Zeller (1938), that the short-chain members of this series were oxidized by histaminase; an optimum rate of oxidation was attained with a 4-carbon chain (putrescine), and the rate of oxidation decreased with increasing chain length. Amine oxidase catalysed the oxidation of long-chain diamines but not that of short-chain diamines.

The present paper contains an account of a study of the homologous series

$$Me_3\overset{+}{N}(CH_2)_n\overset{+}{N}H_3,$$

the series which is intermediate between the bis-onium and bis-amino series. Observations on the oxidation of these compounds as well as on their biological activity are reported.

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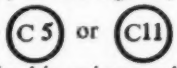
## MATERIAL AND METHODS

*Compounds and Nomenclature.*—In the following, the members of the homologous series studied are briefly referred to as the *hybrids*, e.g., the C5-hybrid or the C11-hybrid; in the figures, they are shown as




The members of the bis-onium series are shown thus: C6 stands for hexamethonium and C10 for decamethonium.

We are grateful to Dr. R. Wien, of Messrs. May & Baker Ltd., for the C6-hybrid, hexane-1-ammonium-6-trimethylammonium bishydrogen tartrate; the synthesis of all the other compounds studied is described below.

*Enzyme Preparations.*—Two preparations with amine oxidase activity were made; one was a homogenate of guinea-pig's liver, the other of rabbit's liver. One gramme of fresh tissue was homogenized and made up to a total volume of 2 ml. with 0.067 M-sodium phosphate buffer of *p*H 7.4. These homogenates were dialysed overnight against tap water; at the end of the dialysis 0.1 ml. of 0.2 M-sodium phosphate buffer of *p*H 7.4 was added to each ml. of dialysate. One ml. of this preparation was usually added to each manometer flask. The compounds were used in an initial concentration of 0.005 M; (−)-*p*-sympatol served as a typical substrate of amine oxidase.

As a source of spermine oxidase, each flask contained 1.6 ml. of ox serum, fully dialysed against the 0.067 M phosphate buffer. As a typical substrate, 0.005 M-spermine tetrahydrochloride was added.

The preparation of histaminase from pig's kidney was as described before (Blaschko and Hawkins, 1950); 0.005 M-cadaverine was the reference substrate.

*Cat's Gastrocnemius.*—This muscle was prepared as described by Bülbring and Burn (1942). Anaesthesia was induced with ether and maintained by an intravenous injection of 80 mg./kg. chloralose. Stimulation of the left sciatic nerve by supramaximal stimuli was by shocks of 0.7 msec. duration at a rate of 8–10 min.

*Preganglionic Stimulation of the Cat's Superior Cervical Ganglion.*—The animals were under chloralose anaesthesia. The contractions of the right nictitating membrane were recorded by a frontal writing lever; magnification of movements was 7.5 times. Electrical stimulation of the cervical sympathetic chain, divided and placed on shielded electrodes and covered with paraffin, was at a rate of 15/sec.; duration of each stimulus was 0.7 msec.

*The rat's phrenic nerve-diaphragm preparation* was set up as described by Bülbring (1946). The diaphragms were suspended in a bath of 75 ml. capacity in Tyrode's solution; oxygenation was by bubbling through a mixture of 95% $O_2$ and 5% $CO_2$; the temperature was 37.5° C.

*The decerebrate pigeon's gastrocnemius* was prepared as described by Ginzel, Klupp, and Werner (1951).

## Chemical Section

*Preparation* of ω-amino-*n*-pentyl,ω-amino-*n*-heptyl, and ω-amino-*n*-decyl trimethylammonium bromide hydrobromides. — Pentamethylene, heptamethylene, and decamethylene dibromides were converted into the corresponding ω-bromoalkyl phthalimides by heating with potassium phthalimide. These were refluxed for 8–12 hr. with a large excess of a 33% w/v solution of trimethylamine in ethanol; the reaction mixture was then concentrated under reduced pressure. The oily residues were redissolved in ethanol, treated with a slight excess of hydrazine hydrate, and made acid to Congo red with dilute (approximately 10%) hydrobromic acid. The phthalyl hydrazide formed was filtered off and the filtrates were concentrated. The resultant gums were dissolved in ethanol and either crystallized from this solvent or were induced to crystallize by addition of methyl ethyl ketone and ether. The yields of pure material were from 20–40%, based on the amounts of bromoalkyl phthalimide.

ω-*Amino*-n-*pentyltrimethylammonium bromide hydrobromide*, recrystallized from ethanol, melted at 221°. Found*: C, 31.2; H, 7.56; N, 9.33. $C_8H_{22}N_2Br_2$ requires C, 31.4; H, 7.26; N, 9.15%.

ω-*Amino*-n-*heptyltrimethylammonium bromide hydrobromide*, recrystallized from ethanol, melted at 224–5°. Found: C, 36.2; H, 7.69; N, 8.30. $C_{10}H_{26}N_2Br_2$ requires C, 35.9; H, 7.79; N, 8.38%.

ω-*Amino*-n-*decyltrimethylammonium bromide hydrobromide*, recrystallized from a mixture of ethanol, methyl ethyl ketone, and ether, sintered at 162° and melted at 164°. Found: C, 41.3; H, 8.24; N, 7.55. $C_{13}H_{32}N_2Br_2$ requires C, 41.5; H, 8.52; N, 7.45%.

*Preparation of* ω-amino-*n*-undecyltrimethylammonium bromide hydrobromide. —ω-Bromoundecylic nitrile (Barlow, 1951) was refluxed for 12 hr. with a large excess of a 33% w/v solution of trimethylamine in ethanol. The reaction mixture was concentrated under reduced pressure and the oily residue dissolved in ethanol saturated with ammonia. This solution was reduced with hydrogen and Raney nickel catalyst at 90 atmospheres and 160°. The catalyst was filtered off, the filtrate concentrated and made acid to Congo red with 50% hydrobromic acid, and then concentrated once more. The residue was dissolved in a mixture of ethanol and methyl ethyl ketone from which the ω-*amino*-n-*undecyltrimethylammonium bromide hydrobromide* crystallized. Recrystallized material sintered at 182° and melted at 185°. Found: C, 43.3; H, 8.69; N, 7.35. $C_{14}H_{34}N_2Br_2$ requires C, 43.1; H, 8.80; N, 7.18%. The yield of pure material was 30%, based on the amount of ω-bromoundecylic nitrile.

*Preparation of* ω-amino-*n*-dodecyltrimethylammonium bromide hydrobromide (with N. A. Dobson).—Ethyl ω-cyanoundecylic ester, obtained in 80% yield from the ω-bromo ester, was reduced with lithium aluminium hydride (1 g./2 g. ester) dissolved in dry ether. When the reaction was over the excess of lithium aluminium hydride was decomposed with water. The mixture was filtered and the ethereal filtrate was used to extract the solid in a Soxhlet apparatus. The ether extract was dried with solid caustic potash and concentrated. The residue was moderately pure ω-amino-*n*-dodecanol and was recrystallized from ether. It melted at 79–80°. Found: C, 71.5; H, 13.4; N, 6.85. $C_{12}H_{27}NO$ requires C, 71.6; H, 13.5; N, 6.97%. The yield was 83%. The substance appears to be a histamine liberator and should be handled with some care.

The amino-alcohol (1 mol.) and phthalic anhydride (1 mol.), dissolved in glacial acetic acid, were heated for 30 min. on a steam-bath. The acetic acid was distilled off under reduced pressure, but the oily residue, presumed to be ω-hydroxy-*n*-dodecyl phthalimide, could not be induced to crystallize. It was heated on a steam-bath for 1 hr. with a slight excess of phosphorus tribromide and a few drops of pyridine. The product was poured into water and the mixture extracted with chloroform. The chloroform extract was dried with anhydrous sodium sulphate and concentrated. The solid residue was recrystallized from petrol (b.p. 60–80°). It melted at 56–58°. Found: C, 61.2; H, 6.85. $C_{20}H_{28}NO_2Br$ requires C, 60.9; H, 7.16%. The yield was 80%.

The ω-bromo-*n*-dodecyl phthalimide was converted into ω-*amino*-n-*dodecyltrimethylammonium bromide hydrobromide* by the method described for the synthesis of the ω-aminopentyl, -heptyl, and -decyl compounds. This was crystallized from a mixture of ethanol and methyl ethyl ketone. It sintered at 200° and melted at 212°. Found: C, 44.4; H, 9.01; N, 6.84. $C_{15}H_{36}N_2Br_2$ requires C, 44.5; H, 8.98; N, 6.93%. The yield of recrystallized material was 30%.

* All analyses are by Mr. J. M. Cameron and and Miss M. M. Christie; M.p.s are uncorrected.

## Results

*Amine Oxidase.*—The compounds of the hybrid series were tested as substrates of amine oxidase. The result of these experiments is shown in Table I.

TABLE I

OXIDATION OF MEMBERS OF THE "HYBRID" SERIES $Me_3\overset{+}{N}(CH_2)_n\overset{+}{N}H_3$ BY AMINE OXIDASE OF GUINEA-PIG AND RABBIT LIVER

The figures are percentages of the rate of oxidation of (−)-*p*-sympatol; they are means of several experiments. Gas: $O_2$; t=37·5° C.

| No. of Methylene Groups *n* | Rate of Oxidation of "Hybrid" Compound as % of Rate of Oxidation of (−)-*p*-sympatol | |
|---|---|---|
| | Guinea-pig Liver | Rabbit Liver |
| 5 | 8 | 8 |
| 6 | 20 | 26 |
| 7 | 12 | 26 |
| 10 | 24 | 55 |
| 11 | 18 | 94 |
| 12 | 41 | 211 |

No significant oxidation of the pentamethylene hybrid was seen, but with increasing chain length the rate of oxygen consumption increased; with both guinea-pig and rabbit liver it was the C12-hybrid that was most rapidly oxidized. That amine oxidase was responsible for the oxidation is supported by the observation that in mixed substrate experiments with (−)-*p*-sympatol the rates of oxidation were not additive, but always intermediate between the rate with the hybrid alone and with (−)-*p*-sympatol alone. This is illustrated by the experiment shown in Fig. 1, where the C11-hybrid was used as substrate and a homogenate of guinea-pig liver as source of enzyme.

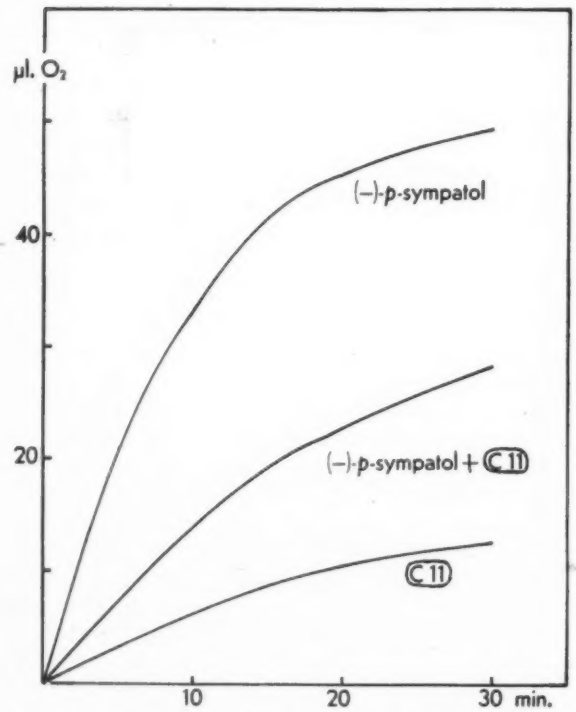



FIG. 1.—Oxidation of the C11-hybrid by a guinea-pig's liver homogenate. The rate of oxidation in the presence of both (−)-*p*-sympatol and C11-hybrid is intermediate between the rates with either substrate alone. Substrate concentrations 0.005M. Gas phase: $O_2$; t=37.5° C.

In an experiment with rabbit liver homogenate, the rates of oxidation of the C10- and C12-hybrids were compared with those of the corresponding members of the bis-onium group. The amounts of oxygen consumed in the first 15 min. in this experiment were:

| | |
|---|---|
| with (−)-*p*-sympatol .. .. | 23 µl. |
| „ the C10-hybrid .. .. | 20 µl. |
| „ decamethylene diamine .. | 43 µl. |
| „ the C12-hybrid .. .. | 53 µl. |
| „ dodecamethylene diamine | 80 µl. |

In other words, the diamine was oxidized at a faster rate than the hybrid compound of the same chain length.

*Spermine Oxidase.*—This enzyme, which has recently been found in the serum of ruminants (Hirsch, 1953), acts not only on spermine but also on other amines. Tabor, Tabor, and Rosenthal (1954) have shown that spermine oxidase also oxidizes decamethylene diamine.

We have found that the enzyme also acts on the C12-hybrid. In an experiment with bovine serum, the amounts of oxygen used in 1 hr. were:

| | |
|---|---|
| with 0.005 M-spermine .. | 128 µl. |
| „ „ -C-12-hybrid .. | 19 µl. |
| „ both amines .. .. | 26 µl. |

This result shows that the hybrid compound, although only slowly oxidized, had a marked effect upon the rate of oxidation of spermine, indicating a high affinity for spermine oxidase.

*Histaminase.*—None of the hybrid compounds was oxidized at a significant rate. When cadaverine was used as a reference substrate, with equimolecular concentrations of either the C5- or the C6-hybrids, the rate of oxidation of cadaverine was not affected, but in the presence of the C7-hybrid cadaverine was not oxidized. With the C10-, C11-, or the C12-hybrids present, the rate of oxidation of cadaverine was about halved.

*Cholinesterases.*—It is known that the bis-onium compounds inhibit acetylcholinesterase (Paton and Zaimis, 1949; Bergmann, Wilson, and Nachmansohn, 1950). The lower members of the hybrid

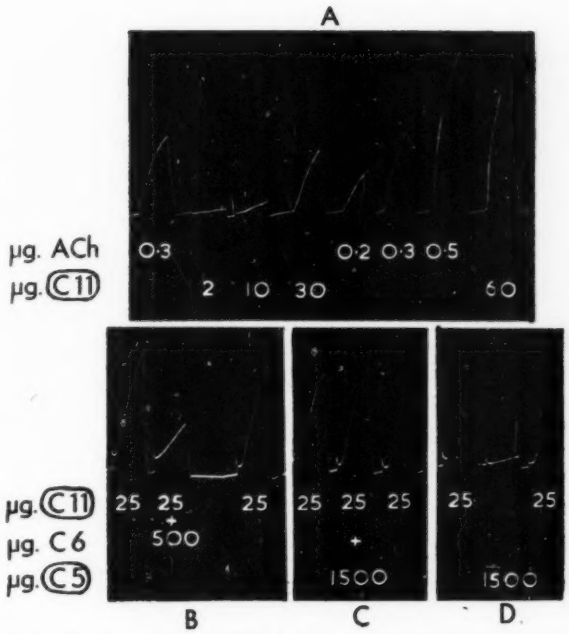



FIG. 2.—Frog's rectus; 1: 100,000 eserine. (A) Comparison of contractures caused by acetylcholine and the C11-hybrid. (B) Effect of hexamethonium on the contracture caused by the C11-hybrid. (C) Effect of the C5-hybrid on the contracture caused by the C11-hybrid. (D) Effect of the C5-hybrid on the muscle.

series had little effect on the hydrolysis of acetyl-β-methylcholine (initial concentration 0.03M) by a preparation of dog's caudate nucleus, but with the C11-hybrid in $10^{-3}$M the rate of hydrolysis was 50% of that without the compound ; with the C12-hybrid in the same concentration it was 45% of the uninhibited rate.

With horse serum, the lower members of the hybrid series did not affect the rate of hydrolysis of 0.06M-benzoylcholine ; with the C11-hybrid the inhibition was 20% ; with the C12-hybrid the inhibition was 90%.

*Frog's Rectus.*—From the work of Paton and Zaimis (1949) it is known that the long-chain members of the bis-onium series (n=10, 11, or 12) cause an acetylcholine-like contracture of this muscle ; the short-chain members (n=5 or 6) are very inactive, but they antagonize the contractions induced by either acetylcholine or decamethonium.

The eserinized frog's rectus muscle was stimulated also by the C11-hybrid. This is shown in Fig. 2a, where the contracture caused by 30 μg. of C11-hybrid was intermediate between that caused by 0.2 μg. and 0.3 μg. respectively of acetylcholine, and the shortening after a dose of 60 μg. of the C11-hybrid equivalent to that after 0.5 μg. of acetylcholine. In this experiment, therefore, acetylcholine was about 120 times as active as the C11-hybrid.

(+)-Tubocurarine, given in a dose of 10 μg., depressed the contracture caused by C11-hybrid, as it depressed the response to acetylcholine. Return of the contracture after tubocurarine occurred slowly.

Whereas there was no qualitative difference in the action of the long-chain members of the hybrid and bis-onium series on the frog's rectus, the C5-hybrid differed from the corresponding bis-onium compound and from hexamethonium in that it had no blocking action on the shortening induced by either acetylcholine or the C11-hybrid (Fig. 2c). Hexamethonium, however, depressed the contracture caused by the C11-hybrid (Fig. 2b). The C5-hybrid, by itself, caused practically no stimulation of the rectus muscle (Fig. 2d).

*Decerebrate Pigeon's Gastrocnemius.*—We are indebted to Dr. K. H. Ginzel for performing the experiment shown in Fig. 3, where an intravenous injection of 10 μg. of decamethonium was given between two injections of 30 μg. each of the C11-hybrid and the C10-hybrid. It can be seen that, like decamethonium, the two compounds of the hybrid series caused a contracture of the pigeon's gastrocnemius muscle. The height of the contractures was of about the same magnitude as that caused by decamethonium, but the shortening with the two hybrids set in very rapidly, and the muscle relaxed again after a shorter interval than after a dose of decamethonium. In a second similar experiment, 1 mg. of the C7-hybrid, given intravenously, caused about the same depression as

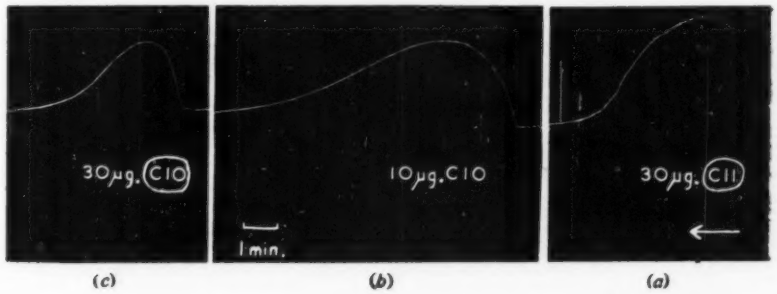



FIG. 3.—Decerebrate pigeon. M. gastrocnemius. Reading from right to left, the figure shows the contractures after intravenous injection of: (*a*) 30 μg. C11-hybrid, (*b*) 10 μg. decamethonium, (*c*) 30 μg. C10-hybrid.

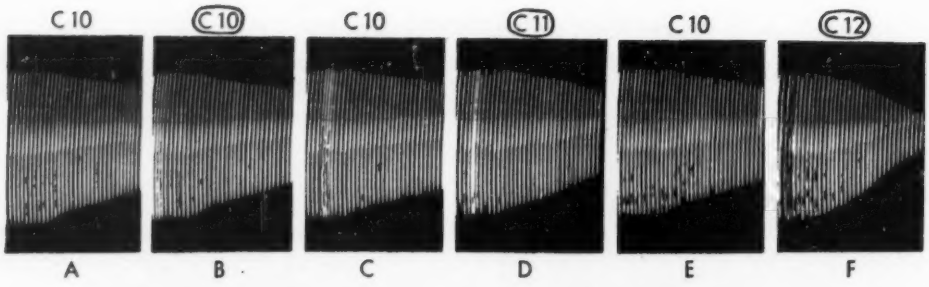
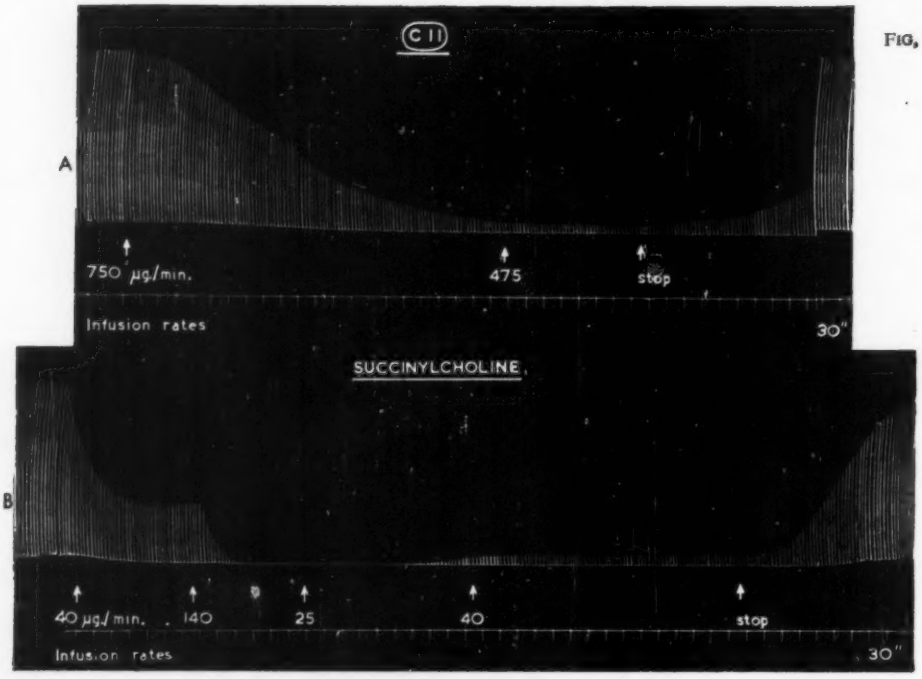



FIG. 4




FIG, 5 A and B

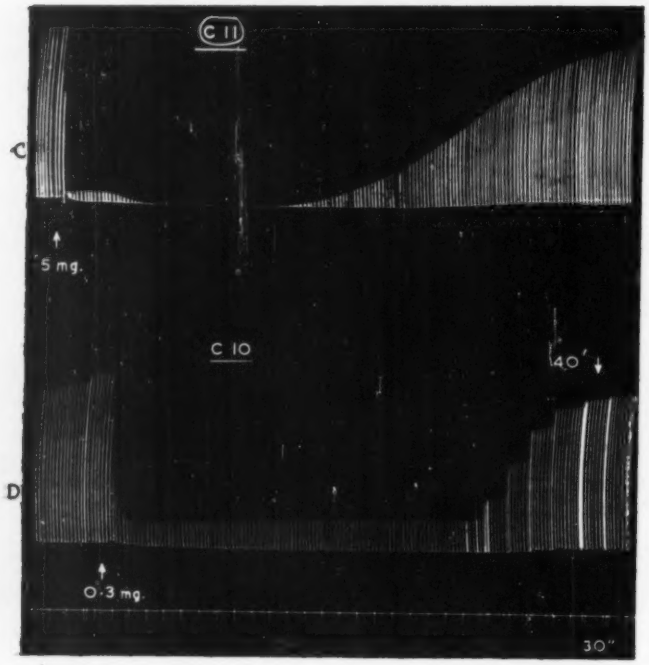



FIG. 5 C and D

FIG. 4.—Rat's phrenic nerve-diaphragm. Each dose was kept in the bath for 3 min.; interval between successive doses 15 min. (A) 1 mg. decamethonium: C10. (B) 1 mg. C10-hybrid: (C 10). (C) 1 mg. decamethonium: C10. (D) 1 mg. C11-hybrid: (C 11). (E) 1 mg. decamethonium: C10. (F) 1 mg. C12-hybrid: (C 12).

FIG. 5.—Cat's gastrocnemius. All tracings were obtained from the same preparation. (A) Intravenous infusion of C11-hybrid was started with 750 µg./min. and later reduced to 475 µg./min. (B) I.v. infusion of succinylcholine; after several changes in the rate of infusion, a depression of contractions similar to that in 5 (A) was obtained with 40 µg./min. (C) Single intravenous dose of 5 mg. C11-hybrid. Note relatively fast recovery. (D) Single i.v. dose of 0.3 mg. decamethonium. Note slow rate of recovery.

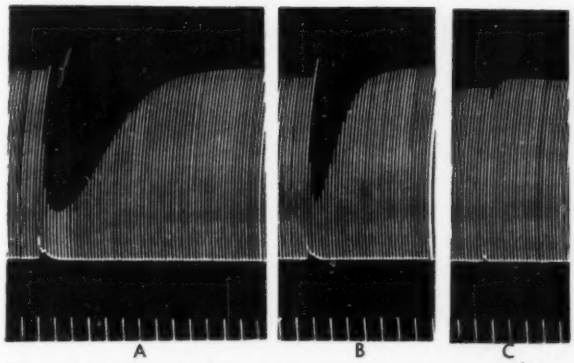



FIG. 6.—Cat's gastrocnemius. Intra-arterial injection of: (A) 200 μg. C10-hybrid, (B) 200 μg. C11-hybrid, and (C) 200 μg. C12-hybrid.

133 μg. of the C10-hybrid; however, the depression came on more gradually and passed off more slowly. An intravenous injection of 20 μg. of decamethonium caused a slightly less pronounced depression, but one which had a time course closely similar to that of the C7-hybrid and very different from the effects of the C10-, C11-, and C12-hybrids with their characteristic rapid onset and rapid recovery.

*Rat's Phrenic Nerve-Diaphragm Preparation.*—Three of the hybrid compounds were tested, those with 10, 11, and 12 methylene groups respectively. All three compounds depressed the response of the muscle to indirect stimulation. The relative potency of the hybrid compounds, in comparison with decamethonium, varied a little from experiment to experiment; in that illustrated in Fig. 4 the C10-hybrid appeared just a little more effective than decamethonium; the blocking activity increased with increasing chain length; the C12-hybrid was the most active compound tested.

The blocking action of the C11-hybrid, like that of decamethonium, was not affected in the presence of neostigmine.

*Cat's Gastrocnemius Muscle.*—In this preparation the long-chain members of the hybrid series also depressed the response to indirect stimulation, but, in contrast to the rat's diaphragm, they were less active than decamethonium.

In one preparation the inhibition of the contractions of the gastrocnemius muscle upon intravenous infusion of the C11-hybrid was compared with that after an infusion of succinylcholine. It was found that the slow infusion of 475 μg./min. of C11-hybrid depressed the response of the muscle to indirect stimulation as strongly as an infusion of 40 μg./min. of succinylcholine. The onset of recovery was slower than after succinylcholine (Fig. 5a and b).

In the same preparation the effects of a single intravenous dose of 300 μg. decamethonium and of 5 mg. of C11-hybrid were also compared; the latter caused an immediate and deep block; however, the effect was transient and the block was complete for only 6 min., and full recovery

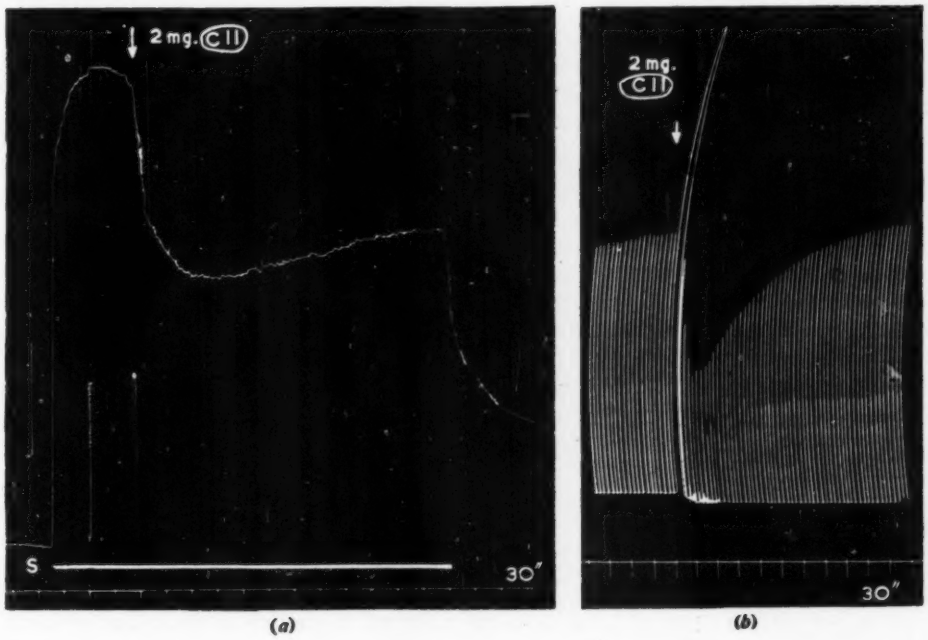



FIG. 7.—Cat; chloralose. Ganglionic blocking action of C11-hybrid. (*a*) Record of contraction of nictitating membrane [stimulated preganglionically at *S*. (*b*) Simultaneous record of gastrocnemius muscle stimulated indirectly.

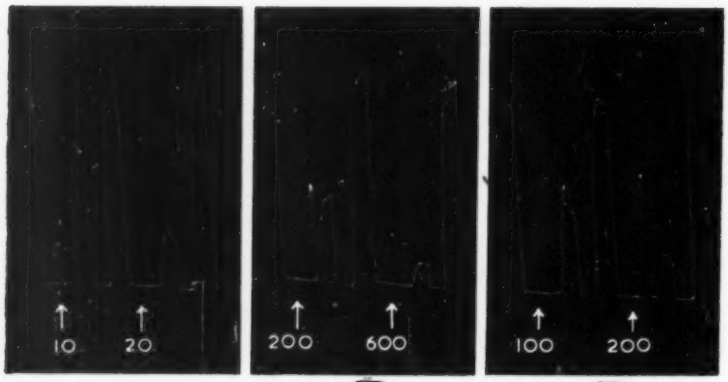



FIG. 8.—Guinea-pig ileum. Capacity of bath, 10 ml. Each contraction is induced by 20 μg. nicotine bitartrate. The following substances were added 30 sec. before the nicotine: at (*a*) hexamethonium, (*b*) C5-hybrid, (*c*) C11-hybrid.

occurred within 15 min. (Fig. 5c). After decamethonium, the block was not complete, but recovery was slower and complete only after 45 min. (Fig. 5d).

The blocking actions of the three long-chain hybrids were compared by intra-arterial injection (Fig. 6). The compounds were given in a dose of 200 μg. The C10-hybrid proved more active than the C11-hybrid, and the C12-hybrid had scarcely any effect in this dose. Given in larger doses, the action of the C12-hybrid was found to be qualitatively similar to that of the deca- and hendecamethylene compounds. Fig. 6 also shows close qualitative similarity between the type of action of the hybrids and those of decamethonium: the depression of the response to indirect stimulation is preceded by a potentiation of the response to indirect stimulation (see also Fig. 7b).

The intravenous injection of 500 mg. iproniazid (Marsilid), an inhibitor of amine oxidase, did not modify the response of the gastrocnemius to the C11-hybrid, neither did the injection of 2.5 mg. tyramine.

*Ganglionic-blocking Action of the C11-hybrid.*—Fig. 7a shows the response, to an intravenous injection of 2 mg. of C11-hybrid, of the preganglionically stimulated nictitating membrane; Fig. 7b shows a simultaneous record of the indirectly stimulated gastrocnemius. It can be seen that the C11-hybrid differs from decamethonium in that it has a ganglionic blocking action which is about as strong as its action upon the neuromuscular junction. The initial excitatory action is clearly shown in Fig. 7b.

*Guinea-pig's Intestine.*—Feldberg (1951) has shown that bis-onium compounds like hexamethonium depress the nicotine-induced contraction of the guinea-pig's intestine. The short-chain members of the hybrid series have only a very feeble activity of this kind. From the experiment shown in Fig. 8, it appears that the C5-hybrid has about one-fifteenth of the activity of hexamethonium; in this preparation the C11-hybrid had a stronger depressor action than the pentamethylene compound.

## DISCUSSION

The members of the series of hybrid compounds were prepared in the first instance in order to test them as possible substrates of amine oxidase. The results of these experiments are in agreement with a picture of amine oxidase as an enzyme with a single receptor for amino groups, whereas histaminase has two. Amine oxidase has no affinity for the short-chain diamines, and this has been explained by the disturbing effect that the second basic group has on the attachment of the first. It is therefore not surprising that the strongly basic onium groups exert a similar disturbing effect. This effect, like that of the amino group in the polymethylene diamines, decreases as the intramolecular distance between the two basic groups increases, and the long-chain members of the hybrid series are therefore oxidized by amine oxidase. A comparison of the rates of oxidation of the C10- and the C12-hybrids with those of the corresponding diamines suggests that the disturbing effect of the onium group is a little more powerful than that of the amino group.

The fact that spermine oxidase, which acts on decamethylene diamine, also oxidizes the C12-hybrid shows the similarity between the specificity requirements of this enzyme and amine oxidase.

Histaminase, which will act on penta- and hexamethylene diamines, does not oxidize the corresponding members of the hybrid series. This is of

interest, because it shows that this enzyme, which requires two basic groups for successful attachment, does not have a significant affinity for compounds in which one of the two amino groups is replaced by an onium group.

The biological activity of the C5-hybrid was compared with that of hexamethonium on the frog's rectus and on the guinea-pig's intestine. On the frog's rectus the compound was without the characteristic depressor action of hexamethonium, and on the guinea-pig's intestine, where the action of hexamethonium is interpreted as a ganglionic-blocking action (Feldberg, 1951), the C5-hybrid is only feebly active. In fact, the long-chain member of the series, the C11-hybrid, was more active upon the intestine than the corresponding pentamethylene derivative, and it is in keeping with this finding that C11-hybrid was also active as blocking agent on the superior cervical ganglion.

On the other hand, the long-chain members of the hybrid series had the characteristic activity of decamethonium on the neuromuscular junction. In most preparations the activity of the hybrids was less, but in the pigeon muscle the ratio was only about one to three, and on the rat's diaphragm the hybrids appeared to be more active than decamethonium.

The responses of striped muscle to the hybrid series were always of much shorter duration than those to decamethonium. On the cat's gastrocnemius the type of response resembled that to succinylcholine. The short duration of the response might be considered as due to destruction of the hybrids by amine oxidase, but this is unlikely. It seems more likely to assume that the amino group, although able to react with the receptor, is less firmly held than the onium group and therefore more easily removed. This is supported by the observation that in the diaphragm, which remains immersed in the fluid containing the hybrid, the potencies of the three hybrids relative to decamethonium were high.

The observations reported show that for the typical activity on the neuromuscular junction one of the two fully methylated onium groups is essential, but the second can be replaced by the primary amino group.

## Summary

1. The synthesis of some members of the "hybrid" series of $\omega$-aminopolymethylene trimethylammonium, $Me_3\overset{+}{N}(CH_2)_n\overset{+}{N}H_3$, is described.

2. The long-chain members of the series are substrates of both amine oxidase and spermine oxidase ; the short-chain members are not oxidized by histaminase.

3. The short-chain members of the hybrid series are without the biological activity of the corresponding members of the bis-onium series.

4. The long-chain members of the hybrid series have an action upon the neuromuscular junction which qualitatively is similar to the action of decamethonium ; in addition they have some ganglionic blocking action.

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# THE ACTIONS OF HETEROCYCLIC BISQUATERNARY COMPOUNDS, ESPECIALLY OF A PYRROLIDINIUM SERIES

BY

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*From May & Baker, Ltd., Dagenham, Essex*

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In a previous paper (Wien and Mason, 1953b) the actions of a series of aromatic bisquaternary compounds were described, and we are now presenting the results on bisquaternary salts containing heterocyclic nuclei, especially of a pyrrolidinium series of compounds. This series was of outstanding interest because of the ganglion-blocking activity of the pentane member, which was about five times as active as hexamethonium. The compounds examined (Libman, Pain, and Slack, 1952) have the general formula $A.(CH_2)_n.A$, where A is a heterocyclic nucleus (1-methylpiperidino, 4-methylmorpholino, and 1-methylpyrrolidino), and from the examination of these and other series it was found that compounds with saturated nuclei were generally more active as ganglion-blocking substances than those with unsaturated nuclei (Wien, 1954b). Some preliminary results on the pyrrolidinium series have already been described (Wien and Mason, 1953a ; Wien, 1954a).

## METHODS

These were the same as described previously (Wien and Mason, 1951) with the following additional procedures:

Perfusion of the superior cervical ganglion was performed (Kibjakow, 1933; Feldberg and Gaddum, 1934) using double dextrose Locke solution containing 1:100,000 eserine for the perfusion fluid, and the acetylcholine in the perfusate was assayed on the blood pressure of the eviscerated cat.

For experiments on parasympathetic ganglia in the whole animal two preparations were used, both in the cat under chloralose anaesthesia. In the first, the salivary flow was recorded from Wharton's duct in response to stimulation of the combined chorda tympani and lingual nerves. Square wave stimuli of 0.4 msec. duration at a rate of 5/sec. were used. In the second preparation the effects on the ciliary ganglion were studied using an extension of the method described by Schofield (1952). The superior cervical ganglion was removed on one side. In the presence of light, and after intravenous injection of the test compound, the pupil on the same side dilated in proportion to the degree of block of the ciliary ganglion. On the opposite side, both the ciliary and superior cervical ganglia were removed. This denervated eye provided a control against the possibility of a direct action of the compound on the iris.

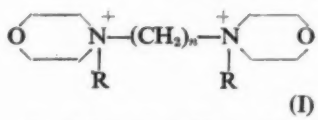
Experiments on neuromuscular transmission were made on the tibialis anterior and soleus muscles (Paton and Zaimis, 1951) as well as on the gastrocnemius of the cat. In addition, the isolated phrenic nerve-diaphragm preparation of the young rabbit was used in some experiments.

Eserine-like properties of the compounds were determined manometrically in the Warburg apparatus at 37° by Ammon's (1933) method, using horse erythrocyte enzyme and acetyl-β-methylcholine as substrate in a final concentration of 0.03 M. The preparation of the enzyme was essentially that of Adams and Thompson (1948).

Our thanks are due to our colleague, Dr. G. Fraser, who carried out the estimations of the anticholinesterase activity.

## RESULTS

### *Morpholinium Series*



$n = 4$ to 12
$X = Br$ or I
$R = Me$ or Et

(I)

In this series (I) we have examined compounds having four to twelve carbon atoms in the chain where R = Me and a compound having six carbon atoms in the chain where R = Et. Where R = Me maximum ganglion-blocking activity was found in the pentane and hexane members. Both were as active as hexamethonium bromide on the nictitating membrane preparation in the cat (on a molar basis they were 1.2 and 1.6 times as active where n = 5 and 6 respectively).

In the single instance where methyl groups on the nitrogen atoms were replaced by ethyl groups,

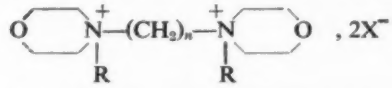
activity was markedly reduced, the resulting compound being only 6% as active as hexamethonium.

No marked neuromuscular-blocking properties were observed in this series ; the compounds where n=9 to 12 only showed 5 to 6% of the activity of (+)-tubocurarine chloride on the sciatic-gastrocnemius preparation in the cat. The decane compound showed some anticholinesterase properties ; on a relative molar basis it had an activity of $6.5 \times 10^{-3}$, where eserine=1.

These results are summarized in Table I.

TABLE I

MORPHOLINIUM SERIES: RELATIVE POTENCIES FOR GANGLION AND NEUROMUSCULAR BLOCK



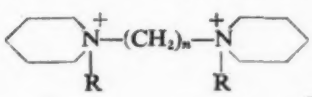
| n | R | X | LD50 mg./g. i.v. (Mice) | Supr. Cerv. Ganglion (Cat) | Gastrocn. (Cat) |
|---|---|---|---|---|---|
| 4 | Me | Br | 0·020 | 0·2 | <0·01 |
| 5 | Me | Br | 0·168 | 1·0 | <0·01 |
| 6 | Me | I | 0·108 | 1·0 | <0·01 |
| 6 | Et | I | 0·060 | 0·06 | <0·01 |
| 7 | Me | I | 0·016 | 0·8 | <0·01 |
| 9 | Me | I | 0·008 | — | 0·05 |
| 10 | Me | I | 0·011 | 0·6 | 0·06 |
| 12 | Me | I | 0 001 | — | 0·06 |
| Hexamethonium bromide | | | 0·050 | 1·0 | — |
| (+)-Tubocurarine chloride | | | 0·0002 | — | 1·0 |

*The hexane compound* (R=Me) had a type of action similar to that of hexamethonium ; for instance, when the effects of preganglionic excitation on the nictitating membrane were completely blocked, post-ganglionic excitation remained unaffected, and the stimulant action of nicotine on the guinea-pig ileum was abolished by a concentration that did not modify the effects of acetylcholine, histamine or pilocarpine.

After administration to rabbits the hexane compound (R=Me) was excreted in the urine to a similar extent to hexamethonium. Thus, both intravenously and subcutaneously, up to 96% was excreted within two days, but orally only 4.4% could be estimated in the urine ; these estimations were by the reineckate method (Zaimis, 1950).

The hexane compound (R=Me) did not release histamine, since in the fully atropinized cat after sufficient nicotine had been injected to block the ganglia intravenous injections of the compound had no effect on the blood pressure. The compound had no anticholinesterase activity and no effect was observed on the salivary secretion stimulated by carbachol. In the conscious cat the intravenous injection of 20 mg./kg. caused marked dilatation of the pupils due to paralysis of the ciliary ganglia, and larger doses, up to 120 mg./kg., depressed respiration but were not fa.al.

*Piperidinium Series*



n = 1 to 10
X = I or Br
R = Me or Et

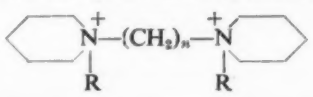
(II)

This series (II) is closely related to the morpholinium series, the oxygen atom in each heterocyclic nucleus being replaced by $-CH_2-$.

Where R=Me, compounds having 3 to 7 and 10 carbon atoms in the chain, and where R=Et, compounds having 6 and 10 carbon atoms, were examined. The results obtained in this series are shown in Table II.

TABLE II

PIPERIDINIUM SERIES: RELATIVE POTENCIES FOR GANGLION AND NEUROMUSCULAR BLOCK



| n | R | X | LD50 mg./g. i.v. (Mice) | Supr. Cerv. Ganglion (Cat) | Gastrocn. (Cat) |
|---|---|---|---|---|---|
| 3 | Me | I | 0 076 | 0·07 | — |
| 4 | Me | I | 0 006 | 0·8 | <0·01 |
| 5 | Me | Br | 0 022 | 0·9 | 0·03 |
| 6 | Me | I | 0·014 | 0·8 | 0·10 |
| 6 | Et | I | 0·011 | <0·1 | 0·10 |
| 7 | Me | I | 0·005 | 0·3 | 0·20 |
| 10 | Me | I | 0·020 | 0·05 | 0·15 |
| 10 | Et | Br | 0·001 | <0·01 | 0·20 |
| Hexamethonium bromide | | | 0·050 | 1·0 | — |
| (+)-Tubocurarine chloride | | | 0·0002 | — | 1·0 |

Where R=Me, three members showed ganglion-blocking properties, where n=4, 5, and 6, and the potency of all three approached that of hexamethonium. On a molar basis the hexane compound was the most active, being 1.25 times as active as hexamethonium. Appreciable neuromuscular-blocking activity was found in the hexane and heptane members as well, but increasing the chain length to decane did not increase this activity. The substitution for methyl by ethyl (where R=Et), in both the hexane and decane members, decreased the ganglion-blocking activity without markedly affecting the neuromuscular-blocking actions.

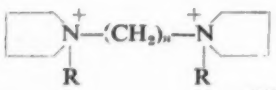
Other actions were displayed by certain members. The ganglion-blocking action of the hexane compound (R=Me) was complicated by slight atropine-like properties. On the salivary flow stimulated by carbachol it was effective at a dose of 1 mg./kg., which is near the dose which produces ganglion block. The decane compound (R=Me) was about one-tenth as active as eserine

against horse red cell enzyme, and in low doses increased the twitch tension of the gastrocnemius.

Neuromuscular-blocking activity of about the same order, it will be observed, occurred in the hexane, heptane and decane members, irrespective of whether the quaternating groups on the nitrogen atoms were methyl or ethyl, and the absence of a marked increase in activity at the decane member characterized this series, as it did also for the morpholinium series. In the morpholinium series, however, none of the compounds showed appreciable neuromuscular-blocking activity.

Several ring substituted compounds (with a chain of 6 carbon atoms) were also examined, in which a methyl group was substituted for a hydrogen atom in both nuclei in either the 2, 3, or 4 positions; all were less active than the parent compound in blocking ganglia, though they retained the same neuromuscular-blocking activity.

*Pyrrolidinium Series*




n = 3 to 12
X = I or Br
R = Me or Et

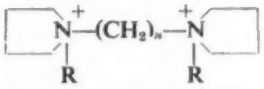
(III)

This series (III) was studied in more detail, particularly the pentane member, which is the most active ganglion-blocking drug we have encountered (Wien and Mason, 1953a; Smirk, 1953; Wien, 1954a).

Pyrrolidine, like piperidine and morpholine, is a saturated ring structure, but is a 5- instead of a 6-membered ring and it is of interest that the pyrrolidine ring occurs in several therapeutic substances. When the experimental work on this series was being carried out, Taylor (1951) independently described the tenth member.

TABLE III

PYRROLIDINIUM SERIES: RELATIVE POTENCIES FOR GANGLION AND NEUROMUSCULAR BLOCK




| n | R | X | LD50 mg./g. i.v. (Mice) | Supr. Cerv. Ganglion (Cat) | Peristalsis (Guinea-pig) | Gastrocn. (Cat) | Diaphragm (Rabbit) |
|---|---|---|---|---|---|---|---|
| 3 | Me | I | 0·127 | 0·05 | 0·1 | <0·1 | <0·1 |
| 4 | Me | I | 0·033 | 0·5 | 0·3 | <0·06 | <0·1 |
| 5 | Me | I | 0·030 | 5·0 | 1·5 | <0·1 | <0·1 |
| 6 | Me | Br | 0·017 | 3·0 | 1·0 | <0·01 | <0·1 |
| 6 | Et | Br | 0·012 | 0·6 | — | — | 0·02 |
| 10 | Me | I | 0·002 | 0·75 | <0·1s | 1·0 | 0·22 |
| 12 | Me | I | 0·003 | 0·10 | 0·5s | 0·25 | 0·01 |
| Hexamethonium bromide | | | 0·050 | 1·0 | 1·0 | — | — |
| (+)-Tubocurarine chloride | | | 0·0002 | | | 1·0 | 1·0 |

s=Stimulant effect on intestine.

Where R=Me we have examined the compounds n=3 to 6, 10 and 12, and where R=Et we only examined the hexane compound. The results are summarized in Table III.

Our interest centred mainly on the pentane and hexane compounds (R=Me), which were very active ganglion-blocking substances, but the decane compound proved also to be a very interesting substance in its neuromuscular-blocking actions. This series was a fruitful source of investigation, for on one hand the pentane compound was several times as active as hexamethonium with a similar type of action, and on the other hand the decane compound was as active as tubocurarine, possessing a rather complex action, mainly resembling that of decamethonium.

*Ganglion Paralysis with Penta- and Hexapyrrolidinium*

"Pentapyrrolidinium," a name which can be applied for convenience to the cation only of the pentane compound, is pentamethylene-1:5-bis(1-methylpyrrolidinium), also known as M&B 2050 (iodide salt) or M&B 2050A and "Ansolysen" (tartrate salt); the approved common name is pentolinium tartrate. This is a white crystalline powder, readily soluble in water; the salt contains 48.6% of base. "Hexapyrrolidinium" (M&B 2024) was examined as the bromide salt.

The relative activities on different ganglia are summarized diagrammatically in Fig. 1, and are considered below in more detail.

*Superior Cervical Ganglion.*—Using the nictitating membrane preparation in the cat and preganglionic excitation, pentapyrrolidinium (iodide), given intravenously, was about five times as active as hexamethonium bromide. This was the mean of several experiments. These results were obtained by matching the observed effect at about a 50% reduction of the maximal contraction of the membrane (Fig. 2); an attempt was made to obtain a more precise figure by using a smaller ratio between the doses and plotting the percentage inhibition of the contraction against the log. dose. The result of such an assay is shown in Fig. 3.

The slopes of the dose-response lines differ, and consequently it is difficult to evaluate precisely one in terms of the other. The duration of the effect of pentapyrrolidinium was slightly longer than that of hexamethonium, a difference which was usually but not always observed. Hexapyrrolidinium was three times as active as hexamethonium on this preparation. The peak of

activity, it will be noted, occurred at the pentane, not the hexane, member as in the methonium series.

*Ciliary Ganglion.*—On this preparation pentapyrrolidinium was slightly more active than it was on the nictitating membrane preparation, but hexapyrrolidinium was equally active on both preparations.

*Salivary Flow.*—In the cat under chloralose, the flow of saliva to chorda-lingual excitation was recorded from Wharton's duct, and the inhibitory effects of compounds injected intravenously were determined. Stimulation was applied continuously during the recording of the effect of a dose, but intervals of 20 min. rest were necessary between doses to allow full recovery (Fig. 4).

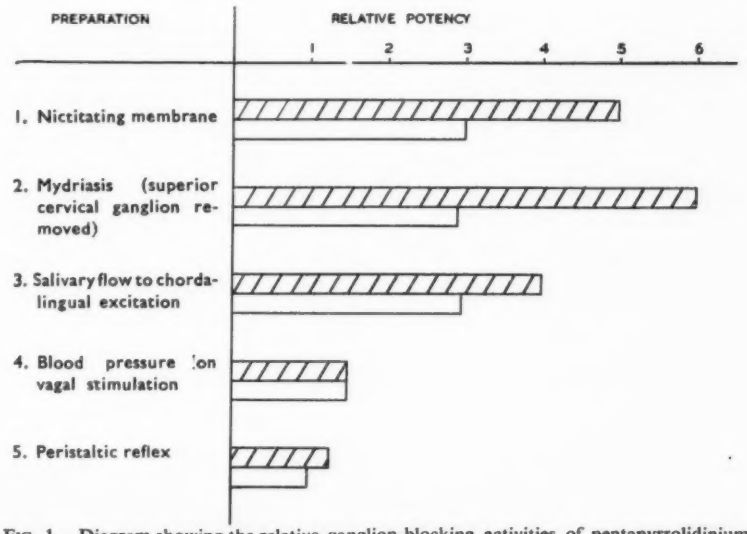



FIG. 1.—Diagram showing the relative ganglion-blocking activities of pentapyrrolidinium (cross-hatched) and hexapyrrolidinium (open) on five different preparations (see text). On each preparation hexamethonium bromide = 1.

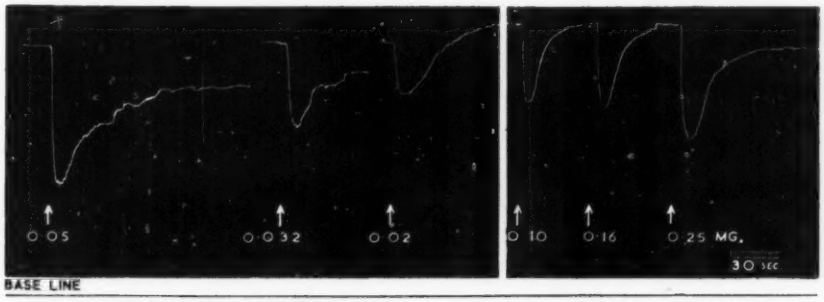



FIG. 2.—Cat, chloralose. Nictitating membrane contractions to sustained preganglionic excitation of the cervical sympathetic nerve. As indicated, intravenous injections in mg. of pentapyrrolidinium (iodide) and hexamethonium (bromide). The data in Fig. 3 were compiled from a similar experiment.

On this preparation, penta- and hexa-pyrrolidinium had similar potencies, relative to hexamethonium, as they had on other preparations.

*Peristaltic Reflex and Cardiac Vagus.*—The two compounds were also examined for ganglion-blocking properties using the peristaltic reflex in isolated guinea-pig ileum, and the bradycardia and fall in blood pressure following stimulation of the peripheral end of the cut vagus in the cat. These methods involve intermittent stimulation, and it was interesting to observe (Fig. 1) that in these two preparations penta- and hexa-pyrrolidinium

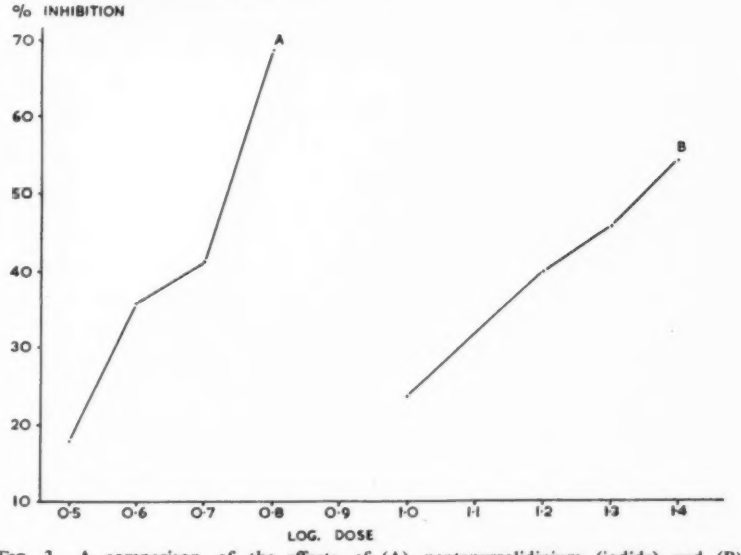



FIG. 3.—A comparison of the effects of (A) pentapyrrolidinium (iodide) and (B) hexamethonium (bromide) on the nictitating membrane preparation in the cat from a single experiment (using sustained preganglionic stimulation), showing the relation of the relaxation of the membrane to intravenous doses of ganglion-blocking agents.

were less active, relative to hexamethonium, than in the three preparations using continuous stimulation.

*Mode of Action*

The mode of action of pentapyrrolidinium was assessed by criteria similar to those used by Paton (1951). Firstly, the ability of the nictitating membrane to contract, and, secondly, the ability of the postganglionic nerves to conduct, were unimpaired during a complete paralysis to preganglionic excitation as shown by the unmodified responses to adrenaline and postganglionic stimulation. Thirdly, during perfusion of the superior cervical ganglion acetylcholine was liberated normally to preganglionic stimulation although the contraction of the nictitating membrane was completely inhibited by injection of the compound into the perfusion fluid. This was observed with doses up to 2 mg., which is several times the dose necessary to block the ganglion. Fourthly, the stimulant effect of nicotine on the guinea-pig ileum was abolished by concentrations of the drug which did not modify the effects of acetylcholine, histamine, and pilocarpine. Fifthly, the effect of stimulation of the peripheral vagus on the blood pressure in the anaesthetized cat was abolished, but the effect of acetylcholine was unmodified.

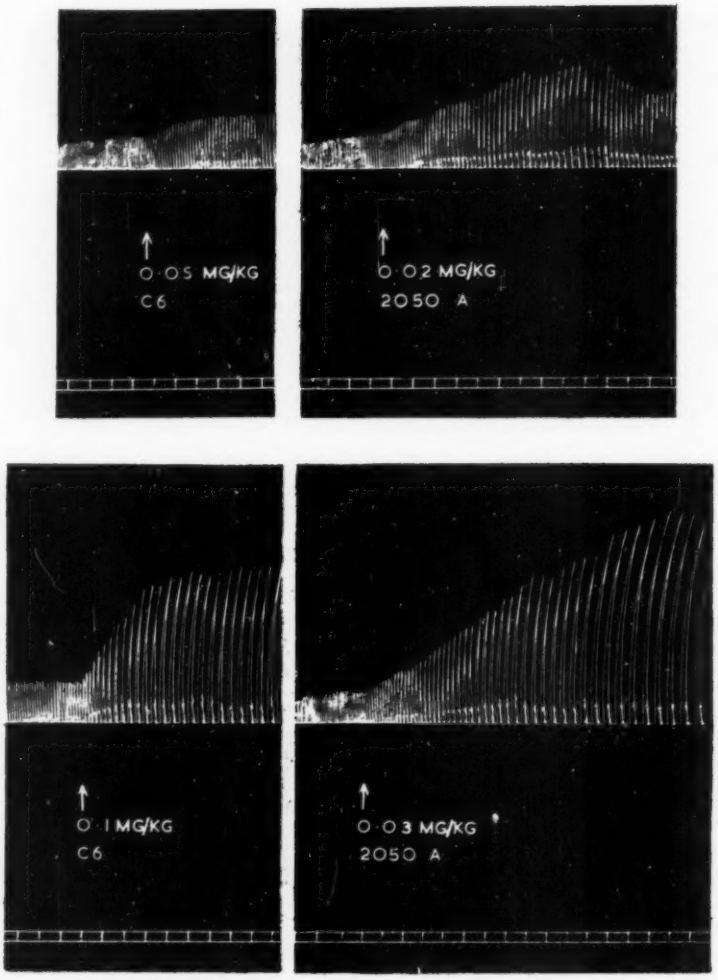



FIG. 4.—Cat, chloralose. Record of the salivary flow from Wharton's duct to electrical stimulation of the chorda tympani at a rate of 5/sec. C6=hexamethonium (bromide) and 2050A=pentapyrrolidinium (bitartrate). Between each dose the preparation was rested for 20 min. Time=30 sec.

Penta- and hexa-pyrrolidinium had no marked atropine-like action; for example, hexapyrrolidinium slightly antagonized salivary flow to infusion of carbachol in an intravenous dose of 10 mg., but this effect was less than that of 4 μg. atropine. There was no release of histamine, as shown by the absence of a depressor action in the atropinized cat under chloralose after ganglionic paralysis with nicotine.

*Toxicity of Pentapyrrolidinium*

*Mice.*—The acute LD50 figures by the intravenous, subcutaneous and oral routes were 0.030 mg./g. (limits of 93–107% for P=0.05, b=20.0), 0.090 mg./g. (limits of 88–114%, b=8.1), and 0.33 mg./g. (limits of 85–119%, b=7.8) respectively. The wide difference between the parenteral and oral toxicities indicated poor gastro-intestinal absorption.

*Rats.*—Daily subcutaneous injections of 0.02 mg./g. for two weeks had no effect on the growth rate of young rats (10 rats in a group). Uninjected controls grew from a mean weight of 52 to 72 g. and the animals in the treated group increased from 52 to 73 g.

*Rabbits.*—Daily intravenous injections of 3 mg./kg. to 6 rabbits over a period of three weeks did not visibly affect the health of any of them. The injections did not cause any local reaction or damage to the veins. Single doses up to 10 mg./kg. produced no obvious marked symptoms, other than slight mydriasis and vasodilatation of the

ear vessels; doses of 20 mg./kg. and more were fatal, and the symptoms seen were paralysis of the neck muscles, dyspnoea and respiratory depression.

*Guinea-pigs.*—A complete blood-picture examination was made by our colleague, Mr. W. A. Freeman, for each of six guinea-pigs at twice-weekly intervals over a period of four weeks during which each animal received daily a subcutaneous injection of 6 mg./kg. No abnormalities were observed. At the end of this period the animals were killed and were kindly examined by Dr. Williamson of the Department of Pathology, Cambridge, who found no pathological changes in any of the tissues except a small localized area of catarrhal inflammation in the lung of one animal. The tissues examined were kidney, lung, bone marrow, heart, liver, suprarenal, spleen, and spinal cord.

*Cats.*—In unanaesthetized cats the intravenous injection (saphena vein) of 5 mg./kg. had no effect; 10 mg./kg. produced only a slight mydriasis, and 20 mg./kg. diminished muscular power; 30 mg./kg. caused pronounced mydriasis, ataxia and depression of respiration, but recovery took place within an hour. The mydriasis lasted for at least an hour after the symptoms of respiratory depression and motor incoordination had passed off.

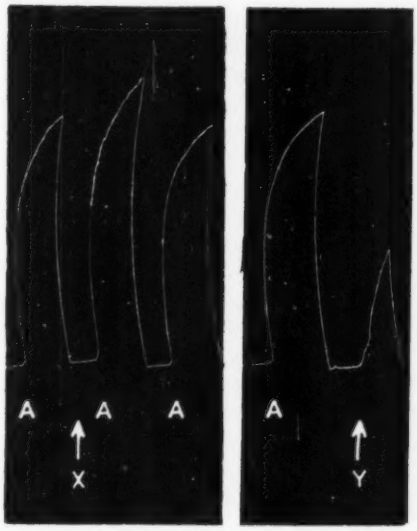



FIG. 5.—Contractions of the isolated frog rectus muscle showing the increased response to acetylcholine (A) in the presence of low concentrations of decapyrrolidinium (X) and the contractions of the muscle to high concentrations of decapyrrolidinium (Y). A=5.0 μg. acetylcholine: X=20 μg. and Y=200 μg. of decapyrrolidinium in a 5 ml. bath.

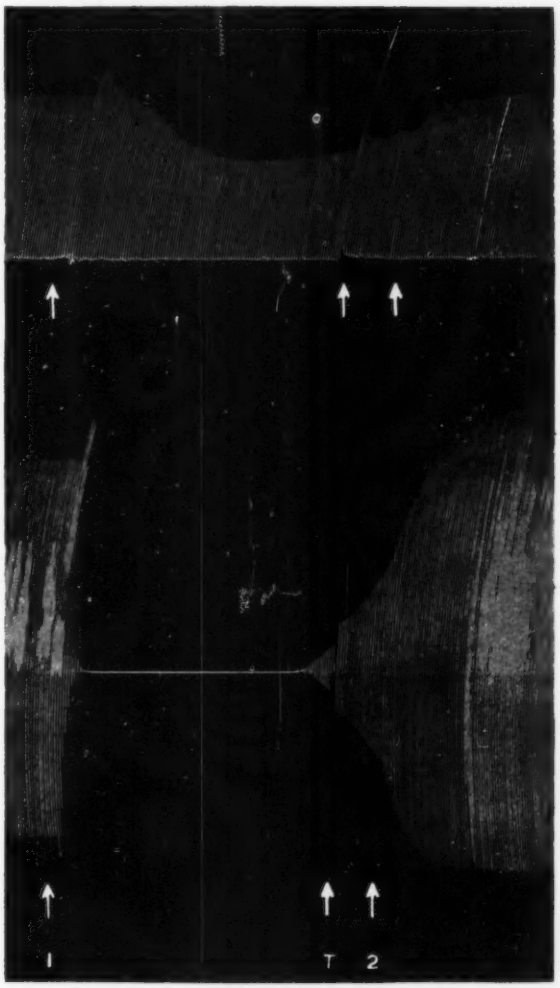



FIG. 6.—Cat, chloralose. Contractions of the soleus (upper record) and tibialis (lower record) to maximal stimulation of the sciatic nerve at a rate of 6/min., 0.5 msec. duration. All injections were intravenous. At (1) 0.5 mg. of decapyrrolidinium; T= tetanus of 50/sec. for 10 sec.; (2) 20 mg. of pentapyrrolidinium.

### *Urinary Excretion of Pentapyrrolidinium in Rabbits*

By the reineckate method the following results were obtained, using 3 rabbits for each route of administration: (*a*) after 15 mg./kg. intravenously, 77.2, 91.3, and 59.3% was eliminated within two days; (*b*) after 30 mg./kg. subcutaneously, 79.0, 74.9, and 82.8% was excreted; and (*c*) after 50 mg./kg. orally 20.1, 23.2, and 19.4% was excreted in the urine.

### *Neuromuscular Paralysis with the Decapyrrolidinium Compound*

The decapyrrolidinium compound had powerful neuromuscular-blocking properties; it reduced the twitch tensions of the gastrocnemius, soleus, and

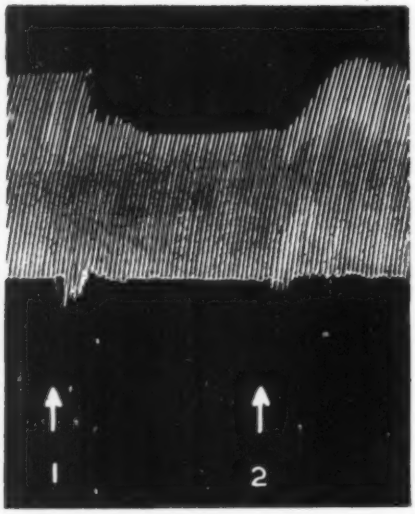



FIG. 7.—Cat, chloralose. Contractions of the gastrocnemius muscle to maximal stimulation of the sciatic nerve at a rate of 6/min., 0.5 msec. duration. All injections were intravenous. At (1) 0.5 mg. decapyrrolidinium and at (2) 0.25 mg. neostigmine injected.

tibialis anterior of the cat to excitation of the sciatic nerve, and of the isolated rabbit diaphragm to excitation of the phrenic nerve. In the anaesthetized cat it was as active as tubocurarine, but its mode of action was complex. In rabbits the head-drop dose by intravenous injection was $0.53 \pm 0.21$ mg./kg. compared with $0.25 \pm 0.04$ mg./kg. for (+)-tubocurarine (8 rabbits on a cross-over test). In conscious cats the induction of paralysis was less smooth than that seen with tubocurarine; initially, marked fasciculations were noted, respiration was quickly depressed and there was salivation. On intravenous injection of 0.1 to 0.5 mg./kg. the duration of the paralysis lasted from 5 to 30 min., and respiration was depressed from 2 to 10 min.; when respiration was arrested a Magill tube was passed under direct vision and artificial respiration maintained.

Most of its effects were like those of decamethonium. In the chick on intravenous injection of 0.1 $\mu$g./g. there was a spastic paralysis and addition of the compound to the isolated frog's rectus abdominis muscle produced a contracture. Moreover, it had an appreciable anti-esterase effect; against the red cell enzyme its molar activity relative to eserine = 1 was $2.3 \times 10^{-2}$, and it potentiated the contractions of acetylcholine on the frog's rectus (Fig. 5).

After an almost complete paralysis of the tibialis anterior muscle in the cat a tetanus was well maintained (Fig. 6), and the muscle twitch was easily restored by an intravenous injection of the pentapyrrolidinium compound, an effect which is analogous to that of the reversal of the action of decamethonium by pentamethonium.

The resemblance to decamethonium was increased by the differential action of decapyrrolidinium on the tibialis and soleus muscles; the tibialis was more readily blocked than the soleus by either decapyrrolidinium or decamethonium (Fig. 6; Paton and Zaimis, 1951).

On the other hand, neostigmine partially reversed a decapyrrolidinium block of the tibialis when in the same experiment it did not reverse a decamethonium block, and a partial paralysis of the gastrocnemius by decapyrrolidinium was also easily reversed by neostigmine. In this way paralysis by decapyrrolidinium resembled that by tubocurarine; the paralysis was accompanied, however, by marked fasciculations (Fig. 7).

## DISCUSSION

Extension of our work now covers a pharmacological study of three series of heterocyclic bisquaternary salts, of which the most interesting was the pyrrolidinium series. Of other series examined it was found that compounds with saturated nuclei of the structure $A.(CH_2)_n.A$, where A was a heterocyclic nucleus, were more active as ganglion-blocking agents than those with unsaturated nuclei (Wien, 1954b). In all three series, morpholinium, piperidinium and pyrrolidinium, replacement of methyl by ethyl groups on the quaternary nitrogen atoms reduced activity in paralysing ganglia.

With the methyl quaternated compounds in the morpholinium series the peak of activity occurred at the pentane and hexane members, in conformity with other observations relating potency and chain length, but the decane member was surprisingly active on ganglia and only feebly active for neuromuscular paralysis. The pentane and hexane compounds were as active as hexamethonium and were less toxic to mice, though the significance of this decreased toxicity is not clear. In so far as these compounds were examined their modes of action were very similar to hexamethonium, and the introduction of heterocyclic nuclei into the cationic head appeared to have made little difference. However, considering the series as a whole, the decane member by contrast was markedly inferior in neuromuscular-blocking potency to decamethonium.

Similarly in the piperidinium series the pentane and hexane members (when the groups on the nitrogens were methyl) were about as active as hexamethonium. In this series, however, mixed

actions were more evident, since the hexane compound had appreciable neuromuscular-blocking properties as well, and the decane compound was a fairly potent inhibitor of cholinesterase. The hexane, heptane, and decane members were all from one-tenth to one-fifth as active as (+)-tubocurarine and in this respect differed from other series, since there was not the sharp differentiation between ganglion- and neuromuscular-blocking properties.

The pyrrolidinium series included several pharmacologically active compounds of potential value as therapeutic substances. The variation in properties on lengthening the chain was very well illustrated in this series.

In all the tests to which the pentane compound was submitted it resembled hexamethonium very closely. Though the fivefold increase in potency was not accompanied by a similar increase in duration of action, there was evidence of a slightly more prolonged effect, and Smirk (1953) has, indeed, found a more prolonged hypotensive action in patients. There was a correspondence between the relative ganglion-blocking values for certain compounds obtained experimentally (on the superior cervical ganglion preparation in the cat), and the ability of these compounds to lower the blood pressure in hypertensive patients. Smirk (1952a, 1952b, and 1953) found that the relative activities in patients for hexamethonium bromide: its bis(ethyldimethyl)analogue: pentapyrrolidinium: and hexapyrrolidinium, were as 1:2:5:1.7 and experimentally in cats these ratios were 1:1.5:5:3. Other clinical work on pentapyrrolidinium has been reported by Hetherington (1953), Freis, Partenope, Lilienfield, and Rose (1954), Rønnov-Jessen (1954), and others.

We have endeavoured to find any selective action that might exist for some of these compounds on different ganglia, and it was considered that the results should preferably be compared in the same species with continuous, rather than intermittent, excitation of the autonomic nervous system. There were three preparations in the cat which roughly fulfilled these conditions (Fig. 1, (1), (2), and (3)). Comparing these results it was found that, relative to hexamethonium, penta- and hexa-pyrrolidinium had about the same order of activity (5:3, 6:3, and 4:3) on each preparation; there was no evidence of a selective action on different ganglia. However, in all these assays there was evidence of some modification of the activity of hexamethonium by pentapyrrolidinium (and vice-versa), which was not completely eliminated even by extended rest periods between doses.

The neuromuscular-blocking properties of the decane compound were interesting; the characteristic features mainly resembled those of a decamethonium block. In the cat under chloralose the maximal twitch of the tibialis was potentiated before the ensuing block, the tibialis was more readily antagonized than the soleus, and the block was not easily, or completely, antagonized by a tetanus or neostigmine; decapyrrolidinium also had anticholinesterase properties. Just as decamethonium can exert a dual mode of action on the muscles, so was there evidence of a variable action with decapyrrolidinium; for example, on the gastrocnemius a partial block could be easily reversed by neostigmine (Fig. 7) resembling block by competition rather than by depolarization. This is not surprising when it has been demonstrated that not only are there species differences in response to neuromuscular-blocking substances, but that differences exist between muscles within any one species (Jewell and Zaimis, 1953). Decapyrrolidinium is another example, like decamethonium and succinylcholine (Zaimis, 1953; Hall and Parkes, 1953), of a compound possessing a dual mode of action.

## Summary

1. The actions are described of three series of heterocyclic bisquaternary salts having the generic formula $A.(CH_2)_n.A$, where A is a heterocyclic nucleus (1-methylpiperidino, 4-methylmorpholino, and 1-methylpyrrolidino).

2. When the groups on the nitrogen atoms were methyl, the butane, pentane, and hexane members of the piperidinium series and the pentane and hexane members of the morpholinium series had ganglion-blocking activity of the same order as hexamethonium. There was only negligible neuromuscular-blocking activity in the morpholinium series; the decane members (either methyl or ethyl groups on the nitrogen atoms) of the piperidinium series were about one-fifth as active as (+)-tubocurarine. The types of action in these two series are described.

3. In the pyrrolidinium series the peak of ganglion-blocking activity occurred at the pentane member (with methyl groups on the nitrogens), which was about five times as active as hexamethonium on the nictitating membrane preparation in the cat.

4. By several tests the mode of action of "pentapyrrolidinium" (pentamethylene-1:5-bis(1-methylpyrrolidinium)) was similar to that of hexamethonium.

5. When examined by experiments in the cat on (i) the salivary flow to chorda-lingual excitation and (ii) the mydriatric response to light (superior cervical ganglion removed), pentapyrrolidinium did not show any marked selective action on either preparation.

6. Decapyrrolidinium was as active as (+)-tubocurarine in producing neuromuscular block in the anaesthetized cat; it had a mixed type of action, which was predominantly decamethonium-like.

We should like to acknowledge the very useful assistance of Mr. G. T. Langston, particularly for carrying out the majority of the experiments on the phrenic-nerve diaphragm preparations. We are indebted to our colleagues, Dr. R. Slack and Dr. H. J. Barber, who devised and synthesized the compounds examined by us.

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# *Chemical Thermodynamics*

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*Translated by*

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